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(71) Applicant: MAX-PLANCK-GESELLSCHAFT ZUR FÖRDERUNG DER WISSENSCHAFTEN E.V. [DE/DE]; Wissenschaften E.V., Bunsenstrasse 10, D-37073 Göttingen (DE).

(72) Inventors: ULLRICH, Axel ; Adalbertstrasse 108, D-80798 München (DE). RISAU, Werner ; Rottenbucherstrasse 54, D-82166 Gräfelfing (DE). MILLAUER, Birgit ; Bodenstedtstrasse 64, D-81241 München (DE).

(74) Agents: RUFF, Michael et al.; Willy-Brandt-Strasse 28, D-70173 Stuttgart (DE).

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FLK-1  866  ILIHIGHHLNVVNLLGACTKPGGPLMVIVEFSKFGNLSTYLRGKRNEFVPYKSKGARFRQ
KDR         -------------------------------C--D-------S----------T------
TKR-C       -------------------------------C---------------------------S

FLK-1  926  GKDYVGELSVDLKRRLDSITSSQSSASSGFVEEKSLSDVEEEEASEELYKDFLTLEHLIC
KDR         ------AIP------------------------------------P-D------------
TKR-C       ------------------------------------------------------------

FLK-1  986  YSFQVAKGMEFLASRKCIHRDLAARNILLSEKNVVKICDFGLARDIYKDPDYVRKGDARL
KDR         ------------------------------------------------------------
TKR-C       ------------------------------------------------------------
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(57) Abstract

The present invention relates to the use of ligands for the Flk-1 receptor for the modulation of angiogenesis and vasculogenesis. The invention is based, in part, on the demonstration that Flk-1 tyrosine kinase receptor expression is associated with endothelial cells and the identification of vascular endothelial growth factor (VEGF) as the high affinity ligand of Flk-1. These results indicate a major role for Flk-1 in the signaling system during vasculogenesis and angiogenesis. Engineering of host cells that express Flk-1 and the uses of expressed Flk-1 to evaluate and screen for drugs and analogs of VEGF involved in Flk-1 modulation by either agonist or antagonist activities is described. The invention also relates to the use of FLK-1 ligands, including VEGF agonists and antagonists, in the treatment of disorders, including cancer, by modulating vasculogenesis and angiogenesis.

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Flk-1 IS A RECEPTOR FOR VASCULAR ENDOTHELIAL GROWTH FACTOR

1. INTRODUCTION

The present invention relates to the use of ligands for the FLK-1 receptor for the modulation of angiogenesis and vasculogenesis. The invention is based, in part, on the demonstration that Flk-1 tyrosine kinase receptor expression is associated with endothelial cells and the identification of vascular endothelial growth factor (VEGF) as the high affinity ligand of Flk-1. These results indicate a major role for Flk-1 in the signaling system during vasculogenesis and angiogenesis. Engineering of host cells that express Flk-1 and the uses of expressed Flk-1 to evaluate and screen for drugs and analogs of VEGF involved in Flk-1 modulation by either agonist or antagonist activities is described.

The invention also relates to the use of FLK-1 ligands, including VEGF agonists and antagonists, in the treatment of disorders, including cancer, by modulating vasculogenesis and angiogenesis.

2. BACKGROUND OF THE INVENTION

Receptor tyrosine kinases comprise a large family of transmembrane receptors for polypeptide growth factors with diverse biological activities. Their intrinsic tyrosine kinase function is activated upon ligand binding, which results in phosphorylation of the receptor and multiple cellular substrates, and subsequently in a variety of cellular responses (Ullrich A. and Schlessinger, J., 1990, Cell 61:203-212).

A receptor tyrosine kinase cDNA, designated fetal liver kinase 1 (Flk-1), was cloned from mouse cell populations enriched for hematopoietic stem and progenitor cells. The receptor was suggested to be involved in hematopoietic stem cell renewal (Matthews

et al., 1991, Proc. Natl. Acad. Sci. USA 88:9026-9030). Sequence analysis of the Flk-1 clone revealed considerable homology with the c-Kit subfamily of receptor kinases and in particular to the *Flt* gene product. These receptors all have in common an extracellular domain containing immunoglobulin-like structures.

The formation and spreading of blood vessels, or vasculogenesis and angiogenesis, respectively, play important roles in a variety of physiological processes such as embryonic development, wound healing, organ regeneration and female reproductive processes such as follicle development in the corpus luteum during ovulation and placental growth after pregnancy. Uncontrolled angiogenesis can be pathological such as in the growth of solid tumors that rely on vascularization for growth.

Angiogenesis involves the proliferation, migration and infiltration of vascular endothelial cells, and is likely to be regulated by polypeptide growth factors. Several polypeptides with in vitro endothelial cell growth promoting activity have been identified. Examples include acidic and basic fibroblastic growth factor, vascular endothelial growth factor and placental growth factor. Although four distinct receptors for the different members of the FGF family have been characterized, none of these have as yet been reported to be expressed in blood vessels in vivo.

While the FGFs appear to be mitogens for a large number of different cell types, VEGF has recently been reported to be an endothelial cell specific mitogen (Ferrara, N. and Henzel, W.J.,1989, Biochem. Biophys. Res. Comm. 161:851-858). Recently, the fms-like tyrosine receptor, *flt*, was shown to have affinity for VEGF (DeVries,C. et al. ,1992, Science 255:989-991).

3. SUMMARY OF THE INVENTION

The present invention relates to the use of ligands for the FLK-1 receptor for the modulation of angiogenesis and vasculogenesis. The present invention is based, in part, on the discovery that the Flk-1 tyrosine kinase receptor is expressed on the surface of endothelial cells and the identification of vascular endothelial growth factor (VEGF) as the high affinity ligand of Flk-1. The role of endothelial cell proliferation and migration during angiogenesis and vasculogenesis indicate an important role for Flk-1 in these processes. The invention is described by way of example for the murine Flk-1, however, the principles may be applied to other species including humans.

Pharmaceutical reagents designed to inhibit the Flk-1/VEGF interaction may be useful in inhibition of tumor growth. VEGF and/or VEGF agonists may be used to promote wound healing. The invention relates to expression systems designed to produce Flk-1 protein and/or cell lines which express the Flk-1 receptor. Expression of soluble recombinant Flk-1 protein may be used to screen peptide libraries for molecules that inhibit the Flk-1/VEGF interaction. Engineered cell lines expressing Flk-1 on their surface may be advantageously used to screen and identify VEGF agonists and antagonists.

4. BRIEF DESCRIPTION OF THE FIGURES

FIG. 1. Comparison of the Flk-1 amino acid sequence with related RTKs. Amino acid sequence comparison of Flk-1 with human KDR and rat TKr-C. A section of the sequence which is known for all three receptors is compared and only differences to the Flk-1 sequence are shown.

FIG. 2. Northern blot analysis of Flk-1 gene expression. (A) Expression of Flk-1 RNA in day 9.5 to day 18.5 mouse embryos. Samples (10 μg) of total RNA from whole mouse embryos were analyzed in each lane. Positions of 28S and 18S ribosomal RNAs are marked. (B) Expression of Flk-1 mRNA in postnatal day 4 and adult brain in comparison with capillary fragments from postnatal day 4 brain. 1μg of poly (A^+) RNA was loaded on each lane. The 5' 2619 bp of the Flk-1 cDNA were used as a probe. Control hybridization with a GAPDH cDNA probe is shown in the lower panel.

FIG. 3. Abundant Flk-1 gene expression in embryonic tissues. *In situ* hybridization analysis of Flk-1 expression in day 14.5 mouse embryo. (A) Bright field illumination of a parasagittal section through the whole embryo hybridized with a ^{35}S-labeled antisense probe (5' 2619 bp). (B) Dark field illumination of the same section. (C) Control hybridization of an adjacent section with a sense probe. Abbreviations: Ao, aorta; At, atrium; L, lung; Li, liver; Ma, mandible; Mn, meninges; Ms. mesencephalon; T, telencephalon; V, ventricle; Vt, vertebrae.

FIG. 4. Expression of Flk-1 RNA in embryonic organs is restricted to specific cells. Expression of Flk-1 RNA in a day 14.5 mouse embryo at higher magnification. (A) The heart region was probed with a ^{35}S-labeled antisense probe. (B) Adjacent section hybridized with the sense probe. (C) Part of the aorta wall shown on the cellular level. The endothelial cell-layer is indicated by an arrow. (D) The lung, probed with the Flk-1 antisense probe. (E) Control hybridization of an adjacent section hybridized with the sense probe. Abbreviations: At, atrium; B, bronchus; Ed, endothelial cell layer; En, endocardium; L, lung, Li,

liver; Lu, lumina of the aorta; Ml, muscular; My, myocardium.

FIG. 5. Flk-1 gene expression in the brain of the developing mouse. *In situ* hybridization analysis of Flk-1 gene expression in the brain at different developmental stages. All sections were probed with the Flk-1 antisense probe. (A) Sagittal section of the telencephalon of a day 11.5 mouse embryo. A single blood vessel expressing Flk-1, which sprouts from the meninges into the neuroectoderm, is indicated by an arrow. (B) Sagittal sections of the brain of embryo day 14.5 and (C) of postnatal day 4. Shown are regions of the mesencephalon. Branching capillaries and blood vessels expressing Flk-1 are indicated by an arrow. (D) Sagittal section of an adult brain; a region of the mesencephalon is shown. Cells expressing Flk-1 are indicated by an arrow. Abbreviations: M, meninges; V, ventricle;

FIG. 6. Expression of Flk-1 in the choroid plexus of adult brain. (A) Darkfield illumination of the choroid plexus of an adult mouse brain hybridized with Flk-1 antisense probe. (B) Choroid plexus shown at a higher magnification. Arrows indicate single cells, which show strong expression of Flk-1. Abbreviations: CP, choroid plexus; E, ependyme; Ep, epithelial cells; V, ventricle.

FIG. 7. Flk-1 is expressed in the glomeruli of the kidney. (A) Parasagittal section of a 4-day postnatal kidney, hybridized with the Flk-1 antisense probe. Hybridization signal accumulates in the glomeruli, as indicated by arrowheads. (B) Control hybridization of an adjacent section with the sense probe. (C) Sagittal section of an adult kidney probed with Flk-1. Arrowheads indicate glomeruli. (D) Glomerulus of an adult kidney at a higher magnification. The arrows in (A) and (D)

indicate cells aligned in strands in the juxtaglomerular region expressing Flk-1.

FIG. 8. *In situ* hybridization analysis of Flk-1 expression in early embryos and extraembryonic tissues. (A) Sagittal section of a day 8.5 mouse embryo in the maternal deciduum probed with Flk-1. (B) Higher magnification of the deciduum. Arrowheads indicate the endothelium of maternal blood vessels strongly expressing Flk-1 RNA. (C) High magnification of the yolk sac and the trophectoderm of a day 9.5 mouse embryo. (D) High magnification of a blood island. Abbreviations: A, allantois; Bi, blood island; Bv, maternal blood vessel; D, deciduum; En, endodermal layer of yolk sac; M, mesenchyme; Ms, mesodermal layer of yolk sac; NF, neural fold; T, trophoblast; Y, yolk sac.

FIG. 9. Flk-1 is a receptor for VEGF. (A) Cross linking of ^{125}I-VEGF to COS cells transiently expressing the Flk-1 receptor and control cells were incubated with ^{125}I-VEGF at 4°C overnight, then washed twice with phosphate buffered saline (PBS) and exposed to 0.5 mM of the cross linking agent DSS in PBS for 1 hour at 4°C. The cells were lysed, Flk-1 receptor immunoprecipitated, and analyzed by polyacrylamide gel electrophoresis followed by autoradiography. Molecular size markers are indicated in kilodaltons. (B) Specific binding of ^{125}I-VEGF to COS cells expressing Flk-1. COS cells transiently expressing Flk-1 were removed from the plate and resuspended in binding medium (DMEM, 25 mM Hepes, 0.15% gelatin). Binding was performed at 15°C for 90 minutes in a total volume of 0.5 ml containing 2×10^5 cells, 15,000 cpm ^{125}I-VEGF, and the indicated concentrations of unlabeled ligand. The cells were washed twice with PBS / 0.1% BSA and counted in a gamma counter.

FIG. 10. VEGF-induced autophosphorylation of Flk-1. COS cells transiently expressing Flk-1 receptor and control cells were starved for 24 hours in DMEM containing 0.5% fetal calf serum and then stimulated with VEGF for 10 minutes as indicated. The cells were solubilized, Flk-1 receptor immunoprecipitated with a polyclonal antibody against its C-terminus, separated by polyacrylamide gel electrophoresis, and transferred to nitrocellulose. The blot was probed with antiphosphotyrosine antibodies (5B2). The protein bands were visualized by using a horseradish-peroxidase coupled secondary antibody and BCL™ (Amersham) detection assay.

FIG. 11. Nucleotide Sequence of Murine Flk-1.

FIG. 12. Plasmid Maps of retroviral vector constructs. pLXSN Flk-1 TM Cl.1 and pLXSN Flk-1 TM cl.3 contain Flk-1 amino acids 1 through 806. pNTK-cfms-TM contains the 541 N-terminal amino acids of c-fms.

FIG. 13. Inhibition of C6 glioblastoma tumor growth by transdominant-negative inhibition of Flk-1. C6 cells were implanted either alone or coimplanted with virus-producing cells. Cell numbers are as indicated in each panel. Two different virus-producing cells lines were used: one expressing the Flk-1 TM (transdominant-negative) mutant and one expressing a transdominant-negative c-fms mutant (c-fms TM) as a control. Beginning at the time when the first tumors appeared, tumor volumes were measured every 2 to 3 days to obtain a growth curve. Each group is represented by four mice.

FIG. 14. Inhibition of C6 glioblastoma tumor growth by transdominant-negative inhibition of Flk-1. C6 cells were implanted either alone or coimplanted with virus-producing cells. Cell numbers are as indicated in each panel. Two different virus-producing cell lines were used: one expressing the Flk-1 TM (transdominant-negative) mutant and one expressing a transdominant-

negative c-fms mutant (cfms TM) as a control. Beginning at the time when the first tumor appeared, tumor volumes were measured every 2 to 3 days to obtain growth curve. Each group is represented by four mice.

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to the use of ligands for the FLK-1 receptor to modulate angiogenesis and/or vasculogenesis. The invention also involves the expression of Flk-1 to evaluate and screen for drugs and analogs of VEGF that may be involved in receptor activation, regulation and uncoupling. Such regulators of Flk-1 may be used therapeutically. For example, agonists of VEGF may be used in processes such as wound healing; in contrast, antagonists of VEGF may be used in the treatment of tumors that rely on vascularization for growth.

The invention, is based, in part, on results from *in situ*-hybridization and Northern blot analyses indicating that Flk-1 is an endothelial cell specific RTK. In addition, cross-linking experiments have shown Flk-1 to be a high affinity receptor for vascular endothelial growth factor (VEGF), indicating that Flk-1 plays a crucial role in the development and differentiation of hemangioblast and in subsequent endothelial cell growth during vasculogenesis and angiogenesis.

The invention is based, also, on the discovery that expression of a transdominant-negative mutant form of the Flk-1 molecule can inhibit the biological activity of the endogenous wild type Flk-1. Experiments are described herein, in which tumor cells and cells producing a recombinant retrovirus encoding a truncated Flk-1 receptor were mixed and injected into mice. Inhibition of vasculogenesis and growth of the injected tumor cells was observed in mice expressing the trucated form of the

Flk-1 receptor. Expression of transdominant negative forms of the Flk-1 molecule may be useful for treatment of diseases resulting from abnormal proliferation of blood vessels, such as rheumatoid arthritis, retinopathies and growth of solid tumors.

As explained in the working examples, infra, the polymerase chain reaction (PCR) method was used to isolate new receptor tyrosine kinases specifically expressed in post-implantation embryos and endothelial cells. One such clone was found to encode a RTK that had almost identical sequence homology with the previously identified cDNA clone isolated from populations of cells enriched for hematopoietic cells and designated fetal liver kinase-1 (Flk-1) (Matthews et al., 1991, Proc. Natl. Acad Sci. U.S.A. 88:9026-9030) (FIG. 11).

For clarity of discussion, the invention is described in the subsections below by way of example for the murine Flk-1. However, the principles may be analogously applied to clone and express the Flk-1 of other species including humans.

5.1. THE Flk-1 CODING SEQUENCE

The nucleotide coding sequence and deduced amino acid sequence of the murine Flk-1 gene is depicted in Figure 11 (SEQ. ID NO. 1) and has recently been described in Matthews et al., 1991, Proc. Natl. Acad. Sci. U.S.A., 88:9026-9030. In accordance with the invention, the nucleotide sequence of the Flk-1 protein or its functional equivalent in mammals, including humans, can be used to generate recombinant molecules which direct the expression of Flk-1; hereinafter, this receptor will be referred to as "Flk-1", regardless of the species from which it is derived.

In a specific embodiment described herein, the murine Flk-1 gene was isolated by performing a polymerase

chain reaction (PCR) using two degenerate oligonucleotide primer pools that were designed on the basis of highly conserved sequences within the kinase domain of receptor tyrosine kinases (Hanks et al., 1988,) As a template, DNA from a λgt10 cDNA library prepared from day 8.5 mouse embryos, was used. In a parallel approach, similar primers were used to amplify RTK cDNA sequences from capillary endothelial cells that had been isolated from the brains of post-natal day 4-8 mice. This is a time when brain endothelial cell proliferation is maximal. Both approaches yielded cDNA sequences encoding the recently described fetal liver RTK, Flk-1 (Matthews et al., 1991). Based on amino acid homology, this receptor is a member of the type III subclass of RTKs (Ullrich and Schlessinger) which contain immunoglobulin-like repeats in their extracellular domains (FIG. 1).

The invention also relates to Flk-1 genes isolated from other species, including humans, in which Flk-1 activity exists. Members of the Flk-1 family are defined herein as those receptors that bind VEGF or fragments of the peptide. Such receptors may demonstrate about 80% homology at the amino acid level in substantial stretches of DNA sequence. A bacteriophage cDNA library may be screened, under conditions of reduced stringency, using a radioactively labeled fragment of the mouse Flk-1 clone. Alternatively the mouse Flk-1 sequence can be used to design degenerate or fully degenerate oligonucleotide probes which can be used as PCR probes or to screen bacteriophage cDNA libraries. A polymerase chain reaction (PCR) based strategy may be used to clone human Flk-1. Two pools of degenerate oligonucleotides, corresponding to a conserved motifs between the mouse Flk-1 and receptor tyrosine kinases, may be designed to serve as primers in a PCR reaction. The template for the reaction is cDNA obtained by reverse transcription of

mRNA prepared from cell lines or tissue known to express human Flk-1. The PCR product may be subcloned and sequenced to insure that the amplified sequences represent the Flk-1 sequences. The PCR fragment may be used to isolate a full length Flk-1 cDNA clone by radioactively labeling the amplified fragment and screening a bacteriophage cDNA library. Alternatively, the labeled fragment may be used to screen a genomic library. For a review of cloning strategies which may be used, see e.g., Maniatis, 1989, Molecular Cloning, A Laboratory Manual, Cold Springs Harbor Press, N.Y.; and Ausubel et al., 1989, Current Protocols in Molecular Biology, (Green Publishing Associates and Wiley Interscience, N.Y.)

Isolation of a human Flk-1 cDNA may also be achieved by construction of a cDNA library in a mammalian expression vector such as pcDNA1, that contains SV40 origin of replication sequences which permit high copy number expression of plasmids when transferred into COS cells. The expression of Flk-1 on the surface of transfected COS cells may be detected in a number of ways, including the use of a labeled ligand such as VEGF or a VEGF agonist labeled with a radiolabel, fluorescent label or an enzyme. Cells expressing the human Flk-1 may be enriched by subjecting transfected cells to a FACS (fluorescent activated cell sorter) sort.

In accordance with the invention, Flk-1 nucleotide sequences which encode Flk-1, peptide fragments of Flk-1, Flk-1 fusion proteins or functional equivalents thereof may be used to generate recombinant DNA molecules that direct the expression of Flk-1 protein or a functionally equivalent thereof, in appropriate host cells. Alternatively, nucleotide sequences which hybridize to portions of the Flk-1 sequence may also be used in

nucleic acid hybridization assays, Southern and Northern blot analyses, etc.

Due to the inherent degeneracy of the genetic code, other DNA sequences which encode substantially the same or a functionally equivalent amino acid sequence, may be used in the practice of the invention for the cloning and expression of the Flk-1 protein. Such DNA sequences include those which are capable of hybridizing to the murine Flk-1 sequence under stringent conditions.

Altered DNA sequences which may be used in accordance with the invention include deletions, additions or substitutions of different nucleotide residues resulting in a sequence that encodes the same or a functionally equivalent gene product. The gene product itself may contain deletions, additions or substitutions of amino acid residues within the Flk-1 sequence, which result in a silent change thus producing a functionally equivalent Flk-1. Such amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipatic nature of the residues involved. For example, negatively charged amino acids include aspartic acid and glutamic acid; positively charged amino acids include lysine and arginine; amino acids with uncharged polar head groups having similar hydrophilicity values include the following: leucine, isoleucine, valine; glycine, analine; asparagine, glutamine; serine, threonine; phenylalanine, tyrosine. As used herein, a functionally equivalent Flk-1 refers to a receptor which binds to VEGF or fragments, but not necessarily with the same binding affinity of its counterpart native Flk-1.

The DNA sequences of the invention may be engineered in order to alter the Flk-1 coding sequence for a variety of ends including but not limited to alterations which modify processing and expression of the gene product.

For example, mutations may be introduced using techniques which are well known in the art, e.g. site-directed mutagenesis, to insert new restriction sites, to alter glycosylation patterns, phosphorylation, etc. For example, in certain expression systems such as yeast, host cells may over glycosylate the gene product. When using such expression systems it may be preferable to alter the Flk-1 coding sequence to eliminate any N-linked glycosylation site.

In another embodiment of the invention, the Flk-1 or a modified Flk-1 sequence may be ligated to a heterologous sequence to encode a fusion protein. For example, for screening of peptide libraries it may be useful to encode a chimeric Flk-1 protein expressing a heterologous epitope that is recognized by a commercially available antibody. A fusion protein may also be engineered to contain a cleavage site located between the Flk-1 sequence and the heterologous protein sequence, so that the Flk-1 can be cleaved away from the heterologous moiety.

In an alternate embodiment of the invention, the coding sequence of Flk-1 could be synthesized in whole or in part, using chemical methods well known in the art. See, for example, Caruthers, et al., 1980, Nuc. Acids Res. Symp. Ser. 7:215-233; Crea and Horn, 180, Nuc. Acids Res. 9(10):2331; Matteucci and Caruthers, 1980, Tetrahedron Letters 21:719; and Chow and Kempe, 1981, Nuc. Acids Res. 9(12):2807-2817. Alternatively, the protein itself could be produced using chemical methods to synthesize the Flk-1 amino acid sequence in whole or in part. For example, peptides can be synthesized by solid phase techniques, cleaved from the resin, and purified by preparative high performance liquid chromatography. (E.g., see Creighton, 1983, Proteins Structures And Molecular Principles, W.H. Freeman and

Co., N.Y. pp. 50-60). The composition of the synthetic peptides may be confirmed by amino acid analysis or sequencing (e.g., the Edman degradation procedure; see Creighton, 1983, Proteins, Structures and Molecular Principles, W.H. Freeman and Co., N.Y., pp. 34-49.

5.2. EXPRESSION OF Flk-1 RECEPTOR AND GENERATION OF CELL LINES THAT EXPRESS Flk-1

In order to express a biologically active Flk-1, the nucleotide sequence coding for Flk-1, or a functional equivalent as described in Section 5.1 supra, is inserted into an appropriate expression vector, i.e., a vector which contains the necessary elements for the transcription and translation of the inserted coding sequence. The Flk-1 gene products as well as host cells or cell lines transfected or transformed with recombinant Flk-1 expression vectors can be used for a variety of purposes. These include but are not limited to generating antibodies (i.e., monoclonal or polyclonal) that bind to the receptor, including those that competitively inhibit binding of VEGF and "neutralize" activity of Flk-1 and the screening and selection of VEGF analogs or drugs that act via the Flk-1 receptor; etc.

5.2.1. EXPRESSION SYSTEMS

Methods which are well known to those skilled in the art can be used to construct expression vectors containing the Flk-1 coding sequence and appropriate transcriptional/translational control signals. These methods include *in vitro* recombinant DNA techniques, synthetic techniques and *in vivo* recombination/genetic recombination. See, for example, the techniques described in Maniatis et al., 1989, Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory, N.Y. and Ausubel et al., 1989, Current Protocols in Molecular

Biology, Greene Publishing Associates and Wiley Interscience, N.Y.

A variety of host-expression vector systems may be utilized to express the Flk-1 coding sequence. These include but are not limited to microorganisms such as bacteria transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing the Flk-1 coding sequence; yeast transformed with recombinant yeast expression vectors containing the Flk-1 coding sequence; insect cell systems infected with recombinant virus expression vectors (e.g., baculovirus) containing the Flk-1 coding sequence; plant cell systems infected with recombinant virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (e.g., Ti plasmid) containing the Flk-1 coding sequence; or animal cell systems infected with recombinant virus expression vectors (e.g., adenovirus, vaccinia virus) including cell lines engineered to contain multiple copies of the Flk-1 DNA either stably amplified (CHO/dhfr) or unstably amplified in double-minute chromosomes (e.g., murine cell lines).

The expression elements of these systems vary in their strength and specificities. Depending on the host/vector system utilized, any of a number of suitable transcription and translation elements, including constitutive and inducible promoters, may be used in the expression vector. For example, when cloning in bacterial systems, inducible promoters such as pL of bacteriophage λ, plac, ptrp, ptac (ptrp-lac hybrid promoter) and the like may be used; when cloning in insect cell systems, promoters such as the baculovirus polyhedrin promoter may be used; when cloning in plant cell systems, promoters derived from the genome of plant cells (e.g., heat shock promoters; the promoter for the

small subunit of RUBISCO; the promoter for the chlorophyll a/b binding protein) or from plant viruses (e.g., the 35S RNA promoter of CaMV; the coat protein promoter of TMV) may be used; when cloning in mammalian cell systems, promoters derived from the genome of mammalian cells (e.g., metallothionein promoter) or from mammalian viruses (e.g., the adenovirus late promoter; the vaccinia virus 7.5K promoter) may be used; when generating cell lines that contain multiple copies of the Flk-1 DNA SV40-, BPV- and EBV-based vectors may be used with an appropriate selectable marker.

In bacterial systems a number of expression vectors may be advantageously selected depending upon the use intended for the Flk-1 expressed. For example, when large quantities of Flk-1 are to be produced for the generation of antibodies or to screen peptide libraries, vectors which direct the expression of high levels of fusion protein products that are readily purified may be desirable. Such vectors include but are not limited to the E. coli expression vector pUR278 (Ruther et al., 1983, EMBO J. 2:1791), in which the Flk-1 coding sequence may be ligated into the vector in frame with the lac Z coding region so that a hybrid AS-lac Z protein is produced; pIN vectors (Inouye & Inouye, 1985, Nucleic acids Res. 13:3101-3109; Van Heeke & Schuster, 1989, J. Biol. Chem. 264:5503-5509); and the like. pGEX vectors may also be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. The pGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned polypeptide of interest can be released from the GST moiety.

In yeast, a number of vectors containing constitutive or inducible promoters may be used. For a review see, Current Protocols in Molecular Biology, Vol. 2, 1988, Ed. Ausubel et al., Greene Publish. Assoc. & Wiley Interscience, Ch. 13; Grant et al., 1987, Expression and Secretion Vectors for Yeast, in Methods in Enzymology, Eds. Wu & Grossman, 1987, Acad. Press, N.Y., Vol. 153, pp. 516-544; Glover, 1986, DNA Cloning, Vol. II, IRL Press, Wash., D.C., Ch. 3; and Bitter, 1987, Heterologous Gene Expression in Yeast, Methods in Enzymology, Eds. Berger & Kimmel, Acad. Press, N.Y., Vol. 152, pp. 673-684; and The Molecular Biology of the Yeast Saccharomyces, 1982, Eds. Strathern et al., Cold Spring Harbor Press, Vols. I and II.

In cases where plant expression vectors are used, the expression of the Flk-1 coding sequence may be driven by any of a number of promoters. For example, viral promoters such as the 35S RNA and 19S RNA promoters of CaMV (Brisson et al., 1984, Nature 310:511-514), or the coat protein promoter of TMV (Takamatsu et al., 1987, EMBO J. 6:307-311) may be used; alternatively, plant promoters such as the small subunit of RUBISCO (Coruzzi et al., 1984, EMBO J. 3:1671-1680; Broglie et al., 1984, Science 224:838-843); or heat shock promoters, e.g., soybean hsp17.5-E or hsp17.3-B (Gurley et al., 1986, Mol. Cell. Biol. 6:559-565) may be used. These constructs can be introduced into plant cells using Ti plasmids, Ri plasmids, plant virus vectors, direct DNA transformation, microinjection, electroporation, etc. For reviews of such techniques see, for example, Weissbach & Weissbach, 1988, Methods for Plant Molecular Biology, Academic Press, NY, Section VIII, pp. 421-463; and Grierson & Corey, 1988, Plant Molecular Biology, 2d Ed., Blackie, London, Ch. 7-9.

An alternative expression system which could be used to express Flk-1 is an insect system. In one such system, Autographa californica nuclear polyhidrosis virus (AcNPV) is used as a vector to express foreign genes. The virus grows in Spodoptera frugiperda cells. The Flk-1 coding sequence may be cloned into non-essential regions (for example the polyhedrin gene) of the virus and placed under control of an AcNPV promoter (for example the polyhedrin promoter). Successful insertion of the Flk-1 coding sequence will result in inactivation of the polyhedrin gene and production of non-occluded recombinant virus (i.e., virus lacking the proteinaceous coat coded for by the polyhedrin gene). These recombinant viruses are then used to infect Spodoptera frugiperda cells in which the inserted gene is expressed. (E.g., see Smith et al., 1983, J. Viol. 46:584; Smith, U.S. Patent No. 4,215,051).

In mammalian host cells, a number of viral based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, the Flk-1 coding sequence may be ligated to an adenovirus transcription/translation control complex, e.g., the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by in vitro or in vivo recombination. Insertion in a non-essential region of the viral genome (e.g., region E1 or E3) will result in a recombinant virus that is viable and capable of expressing Flk-1 in infected hosts. (E.g., See Logan & Shenk, 1984, Proc. Natl. Acad. Sci. (USA) 81:3655-3659). Alternatively, the vaccinia 7.5K promoter may be used. (See, e.g., Mackett et al., 1982, Proc. Natl. Acad. Sci. (USA) 79:7415-7419; Mackett et al., 1984, J. Virol. 49:857-864; Panicali et al., 1982, Proc. Natl. Acad. Sci. 79:4927-4931).

Specific initiation signals may also be required for efficient translation of inserted Flk-1 coding sequences. These signals include the ATG initiation codon and adjacent sequences. In cases where the entire Flk-1 gene, including its own initiation codon and adjacent sequences, is inserted into the appropriate expression vector, no additional translational control signals may be needed. However, in cases where only a portion of the Flk-1 coding sequence is inserted, exogenous translational control signals, including the ATG initiation codon, must be provided. Furthermore, the initiation codon must be in phase with the reading frame of the Flk-1 coding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, etc. (see Bittner et al., 1987, Methods in Enzymol. 153:516-544).

In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Such modifications (e.g., glycosylation) and processing (e.g., cleavage) of protein products may be important for the function of the protein. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins. Appropriate cells lines or host systems can be chosen to ensure the correct modification and processing of the foreign protein expressed. To this end, eukaryotic host cells which possess the cellular machinery for proper processing of the primary transcript, glycosylation, and phosphorylation of the gene product may be used. Such

mammalian host cells include but are not limited to CHO, VERO, BHK, HeLa, COS, MDCK, 293, WI38, etc.

For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines which stably express the Flk-1 may be engineered. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with the Flk-1 DNA controlled by appropriate expression control elements (e.g., promoter, enhancer, sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. Following the introduction of foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. This method may advantageously be used to engineer cell lines which express the Flk-1 on the cell surface, and which respond to VEGF mediated signal transduction. Such engineered cell lines are particularly useful in screening VEGF analogs.

A number of selection systems may be used, including but not limited to the herpes simplex virus thymidine kinase (Wigler, et al., 1977, Cell 11:223), hypoxanthine-guanine phosphoribosyltransferase (Szybalska & Szybalski, 1962, Proc. Natl. Acad. Sci. USA 48:2026), and adenine phosphoribosyltransferase (Lowy, et al., 1980, Cell 22:817) genes can be employed in tk^-, $hgprt^-$ or $aprt^-$ cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for dhfr, which confers resistance to methotrexate (Wigler, et al., 1980, Natl. Acad. Sci. USA 77:3567; O'Hare, et al., 1981, Proc. Natl. Acad. Sci. USA 78:1527); gpt, which confers resistance to

mycophenolic acid (Mulligan & Berg, 1981), Proc. Natl. Acad. Sci. USA 78:2072); neo, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin, et al., 1981, J. Mol. Biol. 150:1); and hygro, which confers resistance to hygromycin (Santerre, et al., 1984, Gene 30:147) genes. Recently, additional selectable genes have been described, namely trpB, which allows cells to utilize indole in place of tryptophan; hisD, which allows cells to utilize histinol in place of histidine (Hartman & Mulligan, 1988, Proc. Natl. Acad. Sci. USA 85:8047); and ODC (ornithine decarboxylase) which confers resistance to the ornithine decarboxylase inhibitor, 2-(difluoromethyl)-DL-ornithine, DFMO (McConlogue L., 1987, In: Current Communications in Molecular Biology, Cold Spring Harbor Laboratory ed.).

5.2.2. IDENTIFICATION OF TRANSFECTANTS OR TRANSFORMANTS THAT EXPRESS THE Flk-1

The host cells which contain the coding sequence and which express the biologically active gene product may be identified by at least four general approaches; (a) DNA-DNA or DNA-RNA hybridization; (b) the presence or absence of "marker" gene functions; (c) assessing the level of transcription as measured by the expression of Flk-1 mRNA transcripts in the host cell; and (d) detection of the gene product as measured by immunoassay or by its biological activity.

In the first approach, the presence of the Flk-1 coding sequence inserted in the expression vector can be detected by DNA-DNA or DNA-RNA hybridization using probes comprising nucleotide sequences that are homologous to the Flk-1 coding sequence, respectively, or portions or derivatives thereof.

In the second approach, the recombinant expression vector/host system can be identified and selected based

upon the presence or absence of certain "marker" gene functions (e.g., thymidine kinase activity, resistance to antibiotics, resistance to methotrexate, transformation phenotype, occlusion body formation in baculovirus, etc.). For example, if the Flk-1 coding sequence is inserted within a marker gene sequence of the vector, recombinants containing the Flk-1 coding sequence can be identified by the absence of the marker gene function. Alternatively, a marker gene can be placed in tandem with the Flk-1 sequence under the control of the same or different promoter used to control the expression of the Flk-1 coding sequence. Expression of the marker in response to induction or selection indicates expression of the Flk-1 coding sequence.

In the third approach, transcriptional activity for the Flk-1 coding region can be assessed by hybridization assays. For example, RNA can be isolated and analyzed by Northern blot using a probe homologous to the Flk-1 coding sequence or particular portions thereof. Alternatively, total nucleic acids of the host cell may be extracted and assayed for hybridization to such probes.

In the fourth approach, the expression of the Flk-1 protein product can be assessed immunologically, for example by Western blots, immunoassays such as radioimmuno-precipitation, enzyme-linked immunoassays and the like. The ultimate test of the success of the expression system, however, involves the detection of the biologically active Flk-1 gene product. A number of assays can be used to detect receptor activity including but not limited to VEGF binding assays; and VEGF biological assays using engineered cell lines as the test substrate.

5.3. USES OF THE Flk-1 RECEPTOR AND ENGINEERED CELL LINES

Angiogenesis, the growth of new blood capillary vessels, is required for a number of physiological processes ranging from wound healing, tissue and organ regeneration, placental formation after pregnancy and embryonic development. Abnormal proliferation of blood vessels is an important component of a variety of diseases such as rheumatoid arthritis, retinopathies, and psoriasis. Angiogenesis is also an important factor in the growth and metastatic activity of solid tumors that rely on vascularization. Therefore, inhibitors of angiogenesis may be used therapeutically for the treatment of diseases resulting from or accompanied by abnormal growth of blood vessels and for treatments of malignancies involving growth and spread of solid tumors.

In an embodiment of the invention the Flk-1 receptor and/or cell lines that express the Flk-1 receptor may be used to screen for antibodies, peptides, or other ligands that act as agonists or antagonists of angiogenesis or vasculogenesis mediated by the Flk-1 receptor. For example, anti-Flk-1 antibodies capable of neutralizing the activity of VEGF, may be used to inhibit Flk-1 function. Additionally, anti-Flk-1 antibodies which mimic VEGF activity may be selected for uses in wound healing. Alternatively, screening of peptide libraries with recombinantly expressed soluble Flk-1 protein or cell lines expressing Flk-1 protein may be useful for identification of therapeutic molecules that function by inhibiting the biological activity of Flk-1.

In an embodiment of the invention, engineered cell lines which express the entire Flk-1 coding region or its ligand binding domain may be utilized to screen and identify VEGF antagonists as well as agonists. Synthetic compounds, natural products, and other sources of

potentially biologically active materials can be screened in a number of ways. The ability of a test compound to inhibit binding of VEGF to Flk-1 may be measured using standard receptor binding techniques, such as those described in Section 6.1.9. The ability of agents to prevent or mimic, the effect of VEGF binding on signal transduction responses on Flk-1 expressing cells may be measured. For example, responses such as activation of Flk-1 kinase activity, modulation of second messenger production or changes in cellular metabolism may be monitored. These assays may be performed using conventional techniques developed for these purposes.

5.3.1. SCREENING OF PEPTIDE LIBRARY WITH Flk-1 PROTEIN OR ENGINEERED CELL LINES

Random peptide libraries consisting of all possible combinations of amino acids attached to a solid phase support may be used to identify peptides that are able to bind to the ligand binding site of a given receptor or other functional domains of a receptor such as kinase domains (Lam, K.S. et al., 1991, Nature 354: 82-84). The screening of peptide libraries may have therapeutic value in the discovery of pharmaceutical agents that act to inhibit the biological activity of receptors through their interactions with the given receptor.

Identification of molecules that are able to bind to the Flk-1 may be accomplished by screening a peptide library with recombinant soluble Flk-1 protein. Methods for expression and purification of Flk-1 are described in Section 5.2.1 and may be used to express recombinant full length Flk-1 or fragments of Flk-1 depending on the functional domains of interest. For example, the kinase and extracellular ligand binding domains of Flk-1 may be separately expressed and used to screen peptide libraries.

To identify and isolate the peptide/solid phase support that interacts and forms a complex with Flk-1, it is necessary to label or "tag" the Flk-1 molecule. The Flk-1 protein may be conjugated to enzymes such as alkaline phosphatase or horseradish peroxidase or to other reagents such as fluorescent labels which may include fluorescein isothyiocynate (FITC), phycoerythrin (PE) or rhodamine. Conjugation of any given label, to Flk-1, may be performed using techniques that are routine in the art. Alternatively, Flk-1 expression vectors may be engineered to express a chimeric Flk-1 protein containing an epitope for which a commercially available antibody exist. The epitope specific antibody may be tagged using methods well known in the art including labeling with enzymes, fluorescent dyes or colored or magnetic beads.

The "tagged" Flk-1 conjugate is incubated with the random peptide library for 30 minutes to one hour at 22°C to allow complex formation between Flk-1 and peptide species within the library. The library is then washed to remove any unbound Flk-1 protein. If Flk-1 has been conjugated to alkaline phosphatase or horseradish peroxidase the whole library is poured into a petri dish containing a substrates for either alkaline phosphatase or peroxidase, for example, 5-bromo-4-chloro-3-indoyl phosphate (BCIP) or 3,3',4,4"-diamnobenzidine (DAB), respectively. After incubating for several minutes, the peptide/solid phase-Flk-1 complex changes color, and can be easily identified and isolated physically under a dissecting microscope with a micromanipulator. If a fluorescent tagged Flk-1 molecule has been used, complexes may be isolated by fluorescent activated sorting. If a chimeric Flk-1 protein expressing a heterologous epitope has been used, detection of the peptide/Flk-1 complex may be accomplished by using a

labeled epitope specific antibody. Once isolated, the identity of the peptide attached to the solid phase support may be determined by peptide sequencing.

In addition to using soluble Flk-1 molecules, in another embodiment, it is possible to detect peptides that bind to cell surface receptors using intact cells. The use of intact cells is preferred for use with receptors that are multi-subunits or labile or with receptors that require the lipid domain of the cell membrane to be functional. Methods for generating cell lines expressing Flk-1 are described in Sections 5.2.1. and 5.2.2. The cells used in this technique may be either live or fixed cells. The cells will be incubated with the random peptide library and will bind to certain peptides in the library to form a "rosette" between the target cells and the relevant solid phase support/peptide. The rosette can thereafter be isolated by differential centrifugation or removed physically under a dissecting microscope.

As an alternative to whole cell assays for membrane bound receptors or receptors that require the lipid domain of the cell membrane to be functional, the receptor molecules can be reconstituted into liposomes where label or "tag" can be attached.

5.3.2. ANTIBODY PRODUCTION AND SCREENING

Various procedures known in the art may be used for the production of antibodies to epitopes of the recombinantly produced Flk-1 receptor. Such antibodies include but are not limited to polyclonal, monoclonal, chimeric, single chain, Fab fragments and fragments produced by an Fab expression library. Neutralizing antibodies i.e., those which compete for the VEGF binding

site of the receptor are especially preferred for diagnostics and therapeutics.

Monoclonal antibodies that bind Flk-1 may be radioactively labeled allowing one to follow their location and distribution in the body after injection. Radioactivity tagged antibodies may be used as a non-invasive diagnostic tool for imaging *de novo* vascularization associated with a number of diseases including rheumatoid arthritis, macular degeneration, and formation of tumors and metastases.

Immunotoxins may also be designed which target cytotoxic agents to specific sites in the body. For example, high affinity Flk-1 specific monoclonal antibodies may be covalently complexed to bacterial or plant toxins, such as diptheria toxin, abrin or ricin. A general method of preparation of antibody/hybrid molecules may involve use of thiol-crosslinking reagents such as SPDP, which attack the primary amino groups on the antibody and by disulfide exchange, attach the toxin to the antibody. The hybrid antibodies may be used to specifically eliminate Flk-1 expressing endothelial cells.

For the production of antibodies, various host animals may be immunized by injection with the Flk-1 protein including but not limited to rabbits, mice, rats, etc. Various adjuvants may be used to increase the immunological response, depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and Corynebacterium parvum.

Monoclonal antibodies to Flk-1 may be prepared by using any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include but are not limited to the hybridoma technique originally described by Kohler and Milstein, (Nature, 1975, 256:495-497), the human B-cell hybridoma technique (Kosbor et al., 1983, Immunology Today, 4:72; Cote et al., 1983, Proc. Natl. Acad. Sci., 80:2026-2030) and the EBV-hybridoma technique (Cole et al., 1985, Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). In addition, techniques developed for the production of "chimeric antibodies" (Morrison et al., 1984, Proc. Natl. Acad. Sci., 81:6851-6855; Neuberger et al., 1984, Nature, 312:604-608; Takeda et al., 1985, Nature, 314:452-454) by splicing the genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity can be used. Alternatively, techniques described for the production of single chain antibodies (U.S. Patent 4,946,778) can be adapted to produce Flk-1-specific single chain antibodies.

Antibody fragments which contain specific binding sites of Flk-1 may be generated by known techniques. For example, such fragments include but are not limited to: the $F(ab')_2$ fragments which can be produced by pepsin digestion of the antibody molecule and the Fab fragments which can be generated by reducing the disulfide bridges of the $F(ab')_2$ fragments. Alternatively, Fab expression libraries may be constructed (Huse et al., 1989, Science, 246:1275-1281) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity to Flk-1.

5.4. USES OF Flk-1 CODING SEQUENCE

The Flk-1 coding sequence may be used for diagnostic purposes for detection of Flk-1 expression. Included in the scope of the invention are oligoribonucleotide sequences, that include antisense RNA and DNA molecules and ribozymes that function to inhibit translation of Flk-1. In addition, mutated forms of Flk-1, having a dominant negative effect, may be expressed in targeted cell populations to inhibit the activity of endogenously expressed wild-type Flk-1.

5.4.1. USE OF Flk-1 CODING SEQUENCE IN DIAGNOSTICS AND THERAPEUTICS

The Flk-1 DNA may have a number of uses for the diagnosis of diseases resulting from aberrant expression of Flk-1. For example, the Flk-1 DNA sequence may be used in hybridization assays of biopsies or autopsies to diagnose abnormalities of Flk-1 expression; e.g., Southern or Northern analysis, including in situ hybridization assays.

The Flk-1 cDNA may be used as a probe to detect the expression of the Flk-1 mRNA. In a specific example described herein, the expression of Flk-1 mRNA in mouse embryos of different developmental stages was analyzed. Northern blot analysis indicated abundant expression of a major 5.5 kb mRNA between day 9.5 and day 18.5, with apparent decline towards the end of gestation (FIG. 2A). In post-natal day 4-8 brain capillaries Flk-1 mRNA was found to be highly enriched compared to total brain RNA (FIG.2B), suggesting a role for Flk-1 in endothelial cell proliferation.

To obtain more detailed information about the expression of Flk-1 during embryonic development and during the early stages of vascular development in situ hybridization experiments were performed as described in Section 6.1.4. In situ hybridizations demonstrated that

Flk-1 expression *in vivo* during embryonic mouse development is largely restricted to endothelial cells and their precursors (FIG. 3 and FIG. 4). Flk-1 is expressed in endothelial cells during physiological processes that are characterized by endothelial cell proliferation and the temporal and spatial expression pattern found in the embryonic brain correlate precisely with the development of the neural vascular system as described by Bar (1980). Vascular sprouts originating in the perineural plexus grow radially into the neuroectoderm and branch there and these sprouts were found to express high amounts of Flk-1 mRNA (FIG. 5). In the early postnatal stages endothelial cell proliferation is still evident and Flk-1 is expressed, whereas in the adult organism, after completion of the vascularization process, the decline in endothelial cell proliferation parallels a decrease in Flk-1 expression.

Also within the scope of the invention are oligoribonucleotide sequences, that include anti-sense RNA and DNA molecules and ribozymes that function to inhibit the translation of Flk-1 mRNA. Anti-sense RNA and DNA molecules act to directly block the translation of mRNA by binding to targeted mRNA and preventing protein translation. In regard to antisense DNA, oligodeoxyribonucleotides derived from the translation initiation site, e.g., between -10 and +10 regions of the Flk-1 nucleotide sequence, are preferred.

Ribozymes are enzymatic RNA molecules capable of catalyzing the specific cleavage of RNA. The mechanism of ribozyme action involves sequence specific hybridization of the ribozyme molecule to complementary target RNA, followed by a endonucleolytic cleavage. Within the scope of the invention are engineered hammerhead motif ribozyme molecules that specifically and

efficiently catalyze endonucleolytic cleavage of Flk-1 RNA sequences.

Specific ribozyme cleavage sites within any potential RNA target are initially identified by scanning the target molecule for ribozyme cleavage sites which include the following sequences, GUA, GUU and GUC. Once identified, short RNA sequences of between 15 and 20 ribonucleotides corresponding to the region of the target gene containing the cleavage site may be evaluated for predicted structural features such as secondary structure that may render the oligonucleotide sequence unsuitable. The suitability of candidate targets may also be evaluated by testing their accessibility to hybridization with complementary oligonucleotides, using ribonuclease protection assays.

Both anti-sense RNA and DNA molecules and ribozymes of the invention may be prepared by any method known in the art for the synthesis of RNA molecules. These include techniques for chemically synthesizing oligodeoxyribonucleotides well known in the art such as for example solid phase phosphoramidite chemical synthesis. Alternatively, RNA molecules may be generated by *in vitro* and *in vivo* transcription of DNA sequences encoding the antisense RNA molecule. Such DNA sequences may be incorporated into a wide variety of vectors which incorporate suitable RNA polymerase promoters such as the T7 or SP6 polymerase promoters. Alternatively, antisense cDNA constructs that synthesize antisense RNA constitutively or inducibly, depending on the promoter used, can be introduced stably into cell lines.

Various modifications to the DNA molecules may be introduced as a means of increasing intracellular stability and half-life. Possible modifications include but are not limited to the addition of flanking sequences of ribo- or deoxy- nucleotides to the 5' and/or 3' ends

of the molecule or the use of phosphorothioate or 2' O-methyl rather than phosphodiesterase linkages within the oligodeoxyribonucleotide backbone.

5.4.2. USE OF DOMINANT NEGATIVE Flk-1 MUTANTS IN GENE THERAPY

Receptor dimerization induced by ligands, is thought to provide an allosteric regulatory signal that functions to couple ligand binding to stimulation of kinase activity. Defective receptors can function as dominant negative mutations by suppressing the activation and response of normal receptors by formation of unproductive heterodimers. Therefore, defective receptors can be engineered into recombinant viral vectors and used in gene therapy in individuals that inappropriately express Flk-1.

In an embodiment of the invention, mutant forms of the Flk-1 molecule having a dominant negative effect may be identified by expression in selected cells. Deletion or missense mutants of Flk-1 that retain the ability to form dimers with wild type Flk-1 protein but cannot function in signal transduction may be used to inhibit the biological activity of the endogenous wild type Flk-1. For example, the cytoplasmic kinase domain of Flk-1 may be deleted resulting in a truncated Flk-1 molecule that is still able to undergo dimerization with endogenous wild type receptors but unable to transduce a signal.

Abnormal proliferation of blood vessels is an important component of a variety of pathogenic disorders such as rheumatoid arthritis, retinopathies and psoriasis. Uncontrolled angiogenesis is also an important factor in the growth and metastases of solid tumors. Recombinant viruses may be engineered to express dominant negative forms of Flk-1 which may be used to

inhibit the activity of the wild type endogenous Flk-1. These viruses may be used therapeutically for treatment of diseases resulting from aberrant expression or activity of Flk-1.

Expression vectors derived from viruses such as retroviruses, vaccinia virus, adeno-associated virus, herpes viruses, or bovine papilloma virus, may be used for delivery of recombinant Flk-1 into the targeted cell population. Methods which are well known to those skilled in the art can be used to construct recombinant viral vectors containing Flk-1 coding sequence. See, for example, the techniques described in Maniatis et al., 1989, Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory, N.Y. and Ausubel et al., 1989, Current Protocols in Molecular Biology, Greene Publishing Associates and Wiley Interscience, N.Y. Alternatively, recombinant Flk-1 molecules can be reconstituted into liposomes for delivery to target cells.

In a specific embodiment of the invention, a deletion mutant of the Flk-1 receptor was engineered into a recombinant retroviral vector. Two clonal isolates designated pLXSN Flk-1 TM cl.1 and pLXSN Flk-1 TM cl.3 contain a truncated Flk-1 receptor lacking the 561 COOH-terminal amino acids. To obtain virus producing cell lines, PA37 cells were transfected with the recombinant vectors and, subsequently, conditioned media containing virus were used to infect GPE cells.

To test whether expression of signaling-defective mutants inhibits endogenous Flk-1 receptor activity, C6 rat gliobastoma cells (tumor cells) and mouse cells producing the recombinant retroviruses were mixed and injected subcutaneously into nude mice. Normally, injection of tumor cells into nude mice would result in proliferation of the tumor cells and vascularization of the resulting tumor mass. Since Flk-1 is believed to be

essential for formation of blood vessels, blocking Flk-1 activity by expression of a truncated receptor, might function to inhibit vascularization of the developing tumor and, thereby, inhibit its growth. As illustrated in Figures 13 and 14, coinjection of virus producing cells, expressing a truncated Flk-1 receptor, significantly inhibits the growth of the tumor as compared to controls receiving only tumor cells.

5.5. USE OF Flk-1 RECEPTOR OR LIGANDS

Receptor/ligand interaction between Flk-1 and VEGF is believed to play an important role in the signalling system during vascularization and angiogenesis. Abnormal proliferation of blood vessels is an important component of a number of diseases.

Expression of Flk-1 RNA correlates with the development of the brain and with endothelial cell proliferation suggesting that Flk-1 might be a receptor involved in mediation of signaling events in the vascularization process. VEGF has been shown to be a mitogenic growth factor known to act exclusively on endothelial cell (Ferrara, N. and Henzel, W.J., 1989, Biochem. Biophys. Res. Comm. 161:851-858). Cross-linking and ligand binding experiments were performed, as described in Section 6.1.9 and 6.1.10 respectively, to determine whether VEGF is a ligand for Flk-1 and the results indicate that Flk-1 is an authentic high affinity VEGF receptor (FIG 9).

In one embodiment of the invention, ligands for Flk-1, the Flk-1 receptor itself, or a fragment containing its VEGF binding site, could be administered <u>in vivo</u> to modulate angiogenesis and/or vasculogenesis. For example, administration of the Flk-1 receptor or a fragment containing the VEGF binding site, could competitively bind to VEGF and inhibit its interaction

with the native Flk-1 receptor in vivo to inhibit angiogenesis and/or vasculogenesis. Alternatively, ligands for Flk-1, including anti-Flk-1 antibodies or fragments thereof, may be used to modulate angiogenesis and/or vasculogenesis. Agonists of VEGF activity may be used to promote wound healing whereas antagonists of VEGF activity may be used to inhibit tumor growth.

Depending on the specific conditions being treated, these agents may be formulated and administered systemically or locally. Techniques for formulation and administration may be found in "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, PA, latest edition. Suitable routes may include oral, rectal, transmucosal, or intestinal administration; parenteral delivery, including intramuscular, subcutaneous, intramedullary injections, as well as intrathecal, direct intraventricular, intravenous, intraperitoneal, intranasal, or intraocular injections, just to name a few. For injection, the agents of the invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks's solution, Ringer's solution, or physiological saline buffer. For such transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

6. EXAMPLE: CLONING AND EXPRESSION PATTERNS OF Flk-1, A HIGH AFFINITY RECEPTOR FOR VEGF

The subsection below describes the cloning and characterization of the Flk-1 cDNA clone. Northern blot and *in situ* hybridization analyses indicate that Flk-1 is expressed in endothelial cells. Cross-linking and ligand binding experiments further indicate that Flk-1 is a high affinity receptor for VEGF.

6.1. MATERIALS AND METHODS

6.1.1. cDNA CLONING OF Flk-1

DNA extracted from λgt10 cDNA library of day 8.5 mouse embryos (Fahrner et al., 1987, EMBO. J. 6:1497-1508) was used as template for polymerase chain reaction (PCR; Saiki, R.K. et al., 1985 Science 230:1350-1354). In an independent approach cDNA of capillary endothelial cells that had been isolated from the brain of postnatal day 4-8 mice was used for amplification (Risau, W., 1990 In: development of the Vascular System. Issues Biomed. Basel Karger 58-68 and Schnürch et al., unpublished) Degenerated primers were designed on the basis of high amino acid homologies within the kinase domain shared by all RTKs (Wilks, A.F., 1989, Proc. Natl. Acad. Sci. U.S.A. 86:1603-1607).

Full length cDNA clones of Flk-1 were isolated from another day 8.5 mouse embryo cDNA library, which had been prepared according to the method of Okayama and Berg (1983), and a day 11.5 mouse embryo λgt11 library (Clonetech) using the ^{32}P-labeled (Feinberg, A.P. and Vogelstein, B. 1983 Anal. Biochem. 132:6-13) 210-bp PCR fragment.

6.1.2. MOUSE EMBRYOS

Balb/c mice were mated overnight and the morning of vaginal plug detection was defined as 1/2 day of gestation. For Northern blot analysis the frozen embryos were homogenized in 5 M guanidinium thiocyanate and RNA was isolated as described (Ullrich, A. et al., 1985, Nature 313:756-761). For *in situ* hybridization, the embryos were embedded in Tissue-Tek (Miles), frozen on the surface of liquid nitrogen and stored at -70C prior to use.

6.1.3. PREPARATION OF PROBES

The 5'-located 2619 bp of the receptor cDNA were subcloned in the pGem3Z vector (Promega) as an EcoR1/BamH1 fragment. The probe for Northern blot hybridization was prepared by labelling the cDNA fragment with α-^{32}PdATP (Amersham) by random hexanucleotide priming (Boehringer; Feinberg, A.P. and Vogelstein, B., 1983 Anal. Biochem. 132:6-13).

For *in situ* hybridization a single-strand antisense DNA probe was prepared as described by Schnürch and Risau (Development, 1991 111:1143-54). The plasmid was linearized at the 3' end of the cDNA and a sense transcript was synthesized using SP6 RNA polymerase (Boehringer). The DNA was degraded using DNAase (RNAase free preparation, Boehringer Mannheim). With the transcript, a random-primed cDNA synthesis with a α-^{35}S dATP (Amersham) was performed by reverse transcription with MMLV reverse transcriptase (BRL). To obtain small cDNA fragments of about 100 bp in average suitable for *in situ* hybridization, a high excess of primer was used. Subsequently the RNA transcript was partially hydrolyzed in 100 mM NaOH for 20 minutes at 70°C, and the probe was neutralized with the same amount of HCl and purified with a Sephadex C50 column. After ethanol precipitation the probe was dissolved at a final specific activity of 5×10^5 cpm. For control hybridization a sense probe was prepared with the same method.

6.1.4. RNA EXTRACTION AND NORTHERN ANALYSIS

Total cytoplasmic RNA was isolated according to the acidic phenol-method of Chromczynski and Sacchi (1987). Poly(A^+) RNA aliquots were electrophoresed in 1.2% agarose formaldehyde (Sambrook, J. et al., 1989 Molecular Cloning: A Laboratory Manual 2nd ed. Cold Spring Harbor Laboratory Press) gels and transferred to nitrocellulose

membranes (Schleicher & Schuell), Hybridizations were performed overnight in 50% formamide, 5 x SSC (750mM sodium chloride, 75mM sodium citrate), 5 x Denhardt's (0.1% Ficoll 400, 0.1% polyvinylpryollidone, 0.1% BSA) and -0.5% SDS at 42°C with 1-3x10^6 cpm-ml^{-1} of ^{32}p-Random primed DNA probe, followed by high stringency washes in 0.2 x SSC, 0.5% SDS at 52°C. The filters were exposed for 4 to 8 days.

6.1.5. *IN SITU* HYBRIDIZATION

Subcloning postfixation and hybridization was essentially performed according to Hogan et al. (1986). 10 µm thick sections were cut at -18°C on a Leitz cryostat. For prehybridization treatment no incubation with 0.2M HCl for removing the basic proteins was performed. Sections were incubated with the ^{35}S-cDNA probe (5x10^4cpm/µl) at 52°C in a buffer containing 50% formamide, 300 mM NuCl, 10 mM Tris-HCl, 10 mM $NaPO_4$ (pH 6.8), 5 mM EDTA, 0.02% Ficoll 400, 0.01% polyvinylprolidone 0.02% BSA 10 m /ml yeast RNA, 10% dextran sulfate, and 10 mM NaCl, 10 mM Tris-HCl, 10 mM $NaPO_4$ (pH 6.8), 5 mM EDTA, 10 Mm DTT at 52°C). For autoradiography, slides were coated with Kodak NTB2 film emulsion and exposed for eight days. After developing, the sections were counterstained and toluidine blue or May-Grinwald.

6.1.6. PREPARATION OF ANTISERA

The 3' primed EcoRV/HindII fragment comprising the 128 C-terminal amino acids of Flk-1 was subcloned in the fusion protein expression vector pGEX3X (Smith, D.B. and Johnson, K.S., 1990 Gene. 67:31-40; Pharmacia). The fusion protein was purified as described and used for immunizing rabbits. After the second boost the rabbits

were bled and the antiserum was used for immunoprecipitation.

6.1.7. TRANSIENT EXPRESSION OF Flk-1 IN COS-1 CELLS

Transfection of COS-1 cells was performed essentially as described by Chen and Okayama (1987 Mol. Cell. Biol. 7:2745-2752) and Gorman et al. (1989 Virology 171:377-385). Briefly, cells were seeded to a density of 1.0×10^6 per 10-cm dish and incubated overnight in DMEM containing 10% fetal calf serum (Gibco). 20 µg of receptor cDNA cloned into a cytomegalovirus promotor driven expression vector was mixed in 0.5 ml of 0.25 M $CaCa_2$, 0.5 ml of 2 x BBS (280 mM NaCl, 1.5 mM Na_2HPO_4, 50 mM BES, pH 6.96 and incubated for 30 min at room temperature. The calcium phosphate/DNA solution was then added to the cells, swirled gently, and incubated for 18 hours at 37°C under 3% CO_2. For ligand binding experiments, the cells were removed from the plate and treated as described below.

To obtain VEGF conditioned media, cells were transfected in 15-cm dishes. Media was collected after 48 h and VEGF was partially purified by affinity chromatography using heparin High Trap TM columns (Pharmacia) and concentrated by ultrafiltration (Ferrara, N. and Henzel, W.J. 1989 Biochem. Biophys. Res. Comm. 161:851-858). The concentration of VEGF was determined by a ligand competition assay with bovine aortic endothelial cells.

For autophosphorylation assays, cells were seeded in 6-well dishes (2×10^5 cells per well), transfected as described above, and starved for 24 h in DMEM containing 0.5% fetal calf serum. The cells were then treated with 500 pM VEGF for 10 min. at 37°C or left untreated and were subsequently lysed as described by Kris et al.

(1985). Flk-1 was immunoprecipitated with an antiserum raised in rabbits against the C-terminus of the receptor. The immunoprecipitates were separated on a 7.5% SDS polyacrylamide gel, transferred to nitrocellulose, and incubated with a mouse monoclonal antibody directed against phosphotyrosine (5E2; Fendly, B.M. et al., 1990 Cancer Research 50:1550-1558). Protein bands were visualized using horseradish peroxidase coupled goat anti-mouse antibody and the ECL™ (Amersham) detection system.

6.1.8. RADIOIODINATION OF VEGF

Recombinant human VEGF (5 μg; generously provided by Dr. H. Weich) was dissolved in 110 μl sodium phosphate buffer pH 76, and iodinated by the procedure of Hunter and Greenwood (1962). The reaction products were separated from the labeled protein by passage over a sephadex G50 column, pre-equilibrated with phosphate buffered saline (PBS) containing 0.7% bovine serum albumin (BSA), and aliquots of the collected fractions were counted before and after precipitation with 20% trichloracetic acid. The purity of the iodinated product was estimated to be superior to 90%, as determined by gel electrophoresis, and the specific activity was 77000 cpm/ng. The bioactivity of the iodinated VEGF was confirmed by comparison with the bioactivities of native VEGF using the tissue factor introduction assay described by Clauss, M. et al. (1990 J. Exp. Med. 172:1535-1545).

6.1.9. CROSSLINKING OF VEGF TO Flk-1

COS-1 cells transiently expressing Flk-1 and untransfected COS-1 cells were incubated with 200 pM ^{125}I-VEGF at 4°C overnight, then washed twice with PBS and exposed to 0.5 mM disuccinimidyl suberate (DSS) in PBS for 1 h at 4°C. The cells were lysed, Flk-1

immunoprecipitated, and analyzed by electrophoresis on a 7% polytarcylamide gel followed by autoradiography.

6.1.10. VEGF BINDING

Ligand binding experiments were performed as described previously (Schumacher, R. et al., 1991, J. Biol. Chem. 266:19288-19295), COS-1 cells were grown in a 15-cm culture dish in DMEM for 48h after transfection. Cells were then washed carefully with PBS and incubated with 5 ml of 25 mM EDTA in PBS for 10 min. Cells were then removed from the plate, washed once with binding buffer (DMEM, 25 mM HEPES, pH 7.5, 0.15% gelatin) and resuspended in 5 ml of binding buffer to determine the cell number. In a total volume of 500 µl this cell suspension was incubated for 90 min at 15°C with 10 pM ^{125}I-VEGF, and increasing concentration of unlabeled ligand (from 0 to 7 x 10^{-9}), which was partially purified from conditioned media of COS-1 cells transiently expressing VEGF (164 amino acid form; Breier et al., 1992). After incubation, cells were washed with PBS 0.1% PBS in the cold. Free ligand was removed by repeated centrifugation and resuspension in binding buffer. Finally, the ^{125}I radioactivity bound to the cells were determined in a gamma counter (Riastar). Data obtained were analyzed by the method of Munson, P.J. and Rodbard, D. (1980 Anal. Biochem. 107:220-235).

6.1.11. RETROVIRAL VECTORS ENCODING TRANSDOMINANT-NEGATIVE MUTANTS OF Flk-1

Recombinant retroviral vectors were constructed that contained the coding region for amino acids 1 through 806 of the Flk-1 receptor (pLX Flk-1 cl.1 and cl.3, Figure 12). A recombinant virus containing a truncated c-fms receptor mutant (pNTK cfms TM cl.7) was used as a control. To obtain virus producing cells mouse GPE cells

were infected with amphotrophic virus-containing conditioned media of PA317 cells that had been transfected with recombinant retroviral DNA. C6 gliobastoma tumor cells were implanted into nude mice either alone or coimplanted with virus producing cells. Injected cell numbers for the two sets of experiments are indicated below. Beginning at the time when the first tumors appeared, tumor volumes were measured every 2 to 3 days to obtain a growth curve.

Experiment No. 1

Number of Mice	Number of C6 Cells	Virus-Producer Cell Line	Number of Virus-Cells
4	5×10^5	pLXSN Flk-1 TM cl.3	1×10^7
4	5×10^5	None	0
4	5×10^5	pNTK cfms TM cl.7	5×10^6

Experiment No. 2

Number of Mice	Number of C6 Cells	Virus-Producer Cell Line	Number of Virus-Cells
4	2×10^6	pLXSN Flk-1 TM cl.1	2×10^7
4	2×10^6	pLXSN Flk-1 TM cl.3	2×10^7
4	2×10^6	None	0
4	2×10^6	pNTK cfms TM cl.7	2×10^7

6.2. RESULTS

6.2.1. ISOLATION OF Flk-1

To identify RTKs that are expressed during mouse development, PCR assays using two degenerate oligonucleotide primer pools that were designed on the basis of highly conserved sequences within the kinase domain of RTKs were performed (Hanks, S.K. et al. 1988, Science 241:42-52). DNA extracted from a λgt10 cDNA library of day 8.5 mouse embryos (Fahrner, K. et al.,

1987, EMBO. J., 6:1497-1508), a stage in mouse development at which many differentiation processes begin was used as the template in the PCR assays. In a parallel approach, with the intention of identifying RTKs that regulate angiogenesis, similar primers were used for the amplification of RTK cDNA sequences from capillary endothelial cells that had been isolated from the brains of postnatal day 4-8 mice, a time at which brain endothelial cell proliferation is maximal (Robertson, P.L. et al., 1985, Devel. Brain Res. 23:219-223). Both approaches yielded cDNA sequences (FIG. 11, SEQ. ID NO.:) encoding the recently described fetal liver RTK, Flk-1 (Matthews, W. et al., 1991, Proc. Natl. Acad. Sci. U.S.A. 88:9026-9030). Based on amino acid homology, this receptor is a member of the type III subclass of RTKs (Ullrich, A. and Schlessinger, J. 1990, Cell 61:203-212) and is closely related to human *flt*, which also contains seven immunoglobin-like repeats in its extracellular domain in contrast to other RTKs of that subfamily, which contain only five such repeat structures (Matthews, W. et al., 1991, Proc. Natl. Acad Sci. U.S.A. 88:9026-9030). Sequence comparisons of Flk-1 with KDR (Terman, B.I. et al., 1991, Oncogene 6:1677-1683) and TKr-C (Sarzani, R. et al., 1992, Biochem. Biophys. Res. Comm. 186:706-714) suggest that these are the human and rat homologues of Flk-1, respectively (Figure 1).

6.2.2 EXPRESSION OF Flk-1 mRNA DURING EMBRYONIC DEVELOPMENT

As a first step towards the elucidation of the biological function of Flk-1, the expression of Flk-1 mRNA was analyzed in mouse embryos at different development stages. Northern blot hybridization experiments indicated abundant expression of a major 5.5 kb mRNA between day 9.5 and day 18.5, with an apparent

decline towards the end of gestation (Figure 2A). In postnatal day 4-8 brain capillaries Flk-1 mRNA was found to be highly enriched compared to total brain mRNA (Figure 2B).

In situ hybridization experiments were performed to obtain more detailed information about the expression of Flk-1 during different embryonal stages. A single-stranded antisense, 2619-nucleotide-long DNA probe comprising the Flk-1 extracellular domain was used as a probe because it generated the most specific hybridization signals. As an example, a parasagittal section of a day 14.5 embryo is shown in Figure 3. High levels of hybridization were detected in the ventricle of the heart, the lung, and the meninges; other tissues such as brain, liver, and mandible appeared to contain fewer cells expressing Flk-1 mRNA. Thin strands of Flk-1 expression were also observed in the intersegmental regions of the vertebrae and at the inner surface of the atrium and the aorta. Higher magnification revealed that the expression of Flk-1 seemed to be restricted to capillaries and blood vessels. Closer examination of the heart, for example, showed positive signals only in the ventricular capillaries and endothelial lining of the atrium (Figure 4A). In the lung, Flk-1 expression was detected in peribronchial capillaries, but was absent from bronchial epithelium (Figure 4D). The aorta showed strong hybridization in endothelial cells, but not in the muscular layer (Figure 4C).

6.2.3. <u>EXPRESSION OF Flk-1 DURING ORGAN ANGIOGENESIS</u>

The neuroectoderm in the telencephalon of a day 11.5 mouse embryo is largely avascular; the first vascular sprouts begin to radially invade the organ originating from the perineural vascular plexus (Bär, J., 1980, Adv. Anat. Embryol. Cell. Biol. 59:1-62; Risau, W. and Lemmon,

V. 1988, Dev. Biol. 125:441-450). At this stage, expression of Flk-1 was high in the perineural vascular plexus and in invading vascular sprouts, as shown in Figure 5A. These in situ hybridization analyses indicated that the proliferating endothelial cells of an angiogenic sprout expressed the Flk-1 mRNA. At day 14.5, when the neuroectoderm is already highly vascularized, numerous radial vessels as well as branching vessels of the intraneural plexus contained large amounts of Flk-1 mRNA (Figure 5B). At postnatal day 4, when sprouting and endothelial cell proliferation is at its highest, strong expression of Flk-1 mRNA was observed in endothelial cells (Figure 5C). Conversely, in the adult brain when angiogenesis has ceased, Flk-1 expression was very low (Figure 5D) and appeared to be restricted mainly to the ehoroid plexus (Figure 6). In the choroid plexus, cells in the inner vascular layer expressed Flk-1 mRNA, while epithelial cells did not (Figure 6A, B).

The embryonic kidney is vascularized by an angiogenic process (Ekblom, P. et al., 1982, Cell Diff. 11:35-39). Glomerular and peritubular capillaries develop synchronously with epithelial morphogenesis. In the postnatal day 4 kidney, in addition to other capillaries, prominent expression of Flk-1 was observed in the presumptive glomerular capillaries (Figure 7A). This expression persisted in the adult kidney (Figure 7C and D) and then seemed to be more confined to the glomerular compared to the early postnatal kidney.

6.2.4. Flk-1 EXPRESSION IN <u>ENDOTHELIAL CELL PROGENITORS</u>

To investigate the possible involvement of Flk-1 in the early stages of vascular development, analysis of embryos at different stages during blood island formation were performed. In a sagittal section of the deciduum of

a day 8.5 mouse embryo, Flk-1 expression was detected on maternal blood vessels in the deciduum, in the yolk sac and in the trophectoderm. Flk-1 mRNA was also found in the allantois and inside the embryo, mainly located in that part where mesenchyma is found (Figure 8A). At a higher magnification of the maternal deciduum, high levels of Flk-1 mRNA expression were found in the inner lining of blood vessels, which consist of endothelial cells (Figure 8B). In the yolk sac, hybridization signals were confined to the mesodermal layer, in which the hemangioblasts differentiate (Figure 8C). Figure 8D shows a blood island at higher magnification, in which the peripheral angioblasts expressed a high level of Flk-1 mRNA.

6.2.5. <u>Flk-1 IS A HIGH AFFINITY RECEPTOR FOR VEGF</u>

Detailed examination of in situ hybridization results and comparison with those for VEGF recently reported by Breier, G. et al. (1992, Development 114:521-532) revealed a remarkable similarity in expression pattern. Furthermore, Flk-1 expression in the glomerular endothelium and VEGF in the surrounding epithelial cells (Breier, G. et al., 1992, Development 114:521-532) raised the possibility of a paracrine relationship between these cells types and suggested therefore a ligand-receptor relationship for VEGF and Flk-1, respectively. In order to test this hypothesis, the full-length Flk-1 cDNA was cloned into the mammalian expression vector pCMV, which contains transcriptional control elements of the human cytomegalovirus (Gorman, C.M. et al., 1989, Virology 171:377-385). For transient expression of the receptor, the Flk-1 expressing plasmid was then transfected into COS-1 fibroblasts.

Specific binding of VEGF to the Flk-1 RTK was demonstrated by crosslinking and competition binding

experiments. Purified ^{125}I-labeled VEGF was incubated with COS-1 cells transfected with the pCMV-Flk-1 expression vector. Crosslinking with DSS and subsequent analysis of immunoprecipitation, PAGE, and autoradiography revealed an approximately 220 kD band which was not detected in the control experiment with untransfected COS-1 cells and is likely to represent the VEGF/Flk-1 receptor complex (Figure 9A). In addition, VEGF competed with ^{125}I-VEGF binding to Flk-1 expressing COS-1 cells (Figure 9B), whereas untransfected COS-1 cells did not bind ^{125}I-VEGF. The interaction of VEGF with the receptor on transfected cells was specific, as PDGF-BB did not compete with binding of ^{125}I-VEGF. Analysis of the binding data revealed a Kd of about 10^{-10} M, suggesting that Flk-1 is a high affinity receptor of VEGF. This finding, together with the Flk-1 and VEGF in situ hybridization results strongly suggests that Flk-1 is a physiologically relevantly receptor for VEGF.

An autophosphorylation assay was performed to confirm the biological relevance of VEGF binding to the Flk-1 receptor. COS1 cells which transiently expressed Flk-1 were starved in DMEM containing 0.5% fetal calf serum for 24h, stimulated with 0.5 mM VEGF, and lysed. The receptors were immunoprecipitated with the Flk-1 specific polyclonal antibody CT128, and then analyzed by SDS-PAGE and subsequent immunoblotting using the antiphosphotyrosine antibody 5E2 (Fendly, B.M. et al., 1990, Cancer Research 50:1550-1558). A shown in Figure 10, VEGF stimulation of Flk-1 expressing cells led to a significant induction of tyrosine phosphorylation of the 180 kD Flk-1 receptor.

6.2.6. INHIBITION OF TUMOR GROWTH BY TRANSDOMINANT-NEGATIVE INHIBITION OF Flk-1

The Flk-1 receptor is believed to play a major role in vasculogenesis and angiogenesis. Therefore, inhibition of Flk-1 activity may inhibit vasculogenesis of a developing tumor and inhibit its growth. To test this hypothesis, tumor cells (C6 rat glioblastoma) and mouse cells producing a recombinant retrovirus encoding a truncated Flk-1 receptor were mixed and implanted subcutaneously into nude mice. The implanted C6 glioblastoma cells secrete VEGF which will bind to and activate the Flk-1 receptors expressed on the surface of mouse endothelial cells. In the absence of any inhibitors of vasculogenesis, the endothelial cells will proliferate and migrate towards the tumor cells. Alternatively, if at the time of injection, the tumor cells are co-injected with cells producing recombinant retrovirus encoding the dominant-negative Flk-1, the endothelial cells growing towards the implanted tumor cells will become infected with recombinant retrovirus which may result in dominant-negative Flk-1 mutant expression and inhibition of endogenous Flk-1 signaling. Suppression of endothelial cell proliferation and migration will result in failure of the implanted tumor cells to become vascularized which will lead to inhibition of tumor growth. As shown in Figures 12 and 13, tumor growth is significantly inhibited in mice receiving implantations of cells producing truncated Flk-1 indicating that expression of a truncated Flk-1 receptor can act in a dominant-negative manner to inhibit the activity of endogenous wild-type Flk-1.

The present invention is not to be limited in scope by the exemplified embodiments which are intended as illustrations of single aspects of the invention, and any clones, DNA or amino acid sequences which are

functionally equivalent are within the scope of the invention. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

It is also to be understood that all base pair sizes given for nucleotides are approximate and are used for purposes of description.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Ullrich, et al

(ii) TITLE OF INVENTION: FIK-1 IS A RECEPTOR FOR VASCULAR ENDOTHELIAL GROWTH FACTOR

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Pennie & Edmonds
(B) STREET: 1155 Avenue of the Americas
(C) CITY: New York
(D) STATE: New York
(E) COUNTRY: U.S.A.
(F) ZIP: 10036-2711

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

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(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Coruzzi, Laura A.
(B) REGISTRATION NUMBER: 30,742
(C) REFERENCE/DOCKET NUMBER: 7683-034-999

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (212) 790-9090
(B) TELEFAX: (212) 869-8864/9741
(C) TELEX: 66141 PENNIE

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5470 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 286..4386

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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ACACCGCTGA CAGCCGCGGC TGGAGCCAGG GCGCCGGTGC CCCGCGCTCT CCCCGGTCTT 180

GCGCTGCGGG GGCCATACCG CCTCTGTGAC TTCTTTGCGG GCCAGGGACG GAGAAGGAGT 240

CTGTGCCTGA GAAACTGGGC TCTGTGCCCA GGCGCGAGGT GCAGG ATG GAG AGC 294
Met Glu Ser
1

AAG GCG CTG CTA GCT GTC GCT CTG TGG TTC TGC GTG GAG ACC CGA GCC 342
Lys Ala Leu Leu Ala Val Ala Leu Trp Phe Cys Val Glu Thr Arg Ala
5 10 15

GCC TCT GTG GGT TTG ACT GGC GAT TTT CTC CAT CCC CCC AAG CTC AGC 390
Ala Ser Val Gly Leu Thr Gly Asp Phe Leu His Pro Pro Lys Leu Ser
20 25 30 35

ACA CAG AAA GAC ATA CTG ACA ATT TTG GCA AAT ACA ACC CTT CAG ATT 438
Thr Gln Lys Asp Ile Leu Thr Ile Leu Ala Asn Thr Thr Leu Gln Ile
40 45 50

ACT TGC AGG GGA CAG CGG GAC CTG GAC TGG CTT TGG CCC AAT GCT CAG 486
Thr Cys Arg Gly Gln Arg Asp Leu Asp Trp Leu Trp Pro Asn Ala Gln
55 60 65

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Arg Asp Ser Glu Glu Arg Val Leu Val Thr Glu Cys Gly Gly Gly Asp
70 75 80

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85 90 95

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Thr Gly Ala Tyr Lys Cys Ser Tyr Arg Asp Val Asp Ile Ala Ser Thr
100 105 110 115

GTT TAT GTC TAT GTT CGA GAT TAC AGA TCA CCA TTC ATC GCC TCT GTC 678
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120 125 130

AGT GAC CAG CAT GGC ATC GTG TAC ATC ACC GAG AAC AAG AAC AAA ACT 726
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150 155 160

TGC GCT AGG TAT CCA GAA AAG AGA TTT GTT CCG GAT GGA AAC AGA ATT 822
Cys Ala Arg Tyr Pro Glu Lys Arg Phe Val Pro Asp Gly Asn Arg Ile
165 170 175

TCC TGG GAC AGC GAG ATA GGC TTT ACT CTC CCC AGT TAC ATG ATC AGC 870
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Tyr Ala Gly Met Val Phe Cys Glu Ala Lys Ile Asn Asp Glu Thr Tyr
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Gln Ser Ile Met Tyr Ile Val Val Val Val Gly Tyr Arg Ile Tyr Asp
215 220 225

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230 235 240

CTT GTC TTA AAT TGT ACA GCG AGA ACA GAG CTC AAT GTG GGG CTT GAT 1062
Leu Val Leu Asn Cys Thr Ala Arg Thr Glu Leu Asn Val Gly Leu Asp
245 250 255

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TTC ACC TGG CAC TCT CCA CCT TCA AAG TCT CAT CAT AAG AAG ATT GTA   1110
Phe Thr Trp His Ser Pro Pro Ser Lys Ser His His Lys Lys Ile Val
260                 265                 270                 275

AAC CGG GAT GTG AAA CCC TTT CCT GGG ACT GTG GCG AAG ATG TTT TTG   1158
Asn Arg Asp Val Lys Pro Phe Pro Gly Thr Val Ala Lys Met Phe Leu
            280                 285                 290

AGC ACC TTG ACA ATA GAA AGT GTG ACC AAG AGT GAC CAA GGG GAA TAC   1206
Ser Thr Leu Thr Ile Glu Ser Val Thr Lys Ser Asp Gln Gly Glu Tyr
        295                 300                 305

ACC TGT GTA GCG TCC AGT GGA CGG ATG ATC AAG AGA AAT AGA ACA TTT   1254
Thr Cys Val Ala Ser Ser Gly Arg Met Ile Lys Arg Asn Arg Thr Phe
    310                 315                 320

GTC CGA GTT CAC ACA AAG CCT TTT ATT GCT TTC GGT AGT GGG ATG AAA   1302
Val Arg Val His Thr Lys Pro Phe Ile Ala Phe Gly Ser Gly Met Lys
    325                 330                 335

TCT TTG GTG GAA GCC ACA GTG GGC AGT CAA GTC CGA ATC CCT GTG AAG   1350
Ser Leu Val Glu Ala Thr Val Gly Ser Gln Val Arg Ile Pro Val Lys
340                 345                 350                 355

TAT CTC AGT TAC CCA GCT CCT GAT ATC AAA TGG TAC AGA AAT GGA AGG   1398
Tyr Leu Ser Tyr Pro Ala Pro Asp Ile Lys Trp Tyr Arg Asn Gly Arg
            360                 365                 370

CCC ATT GAG TCC AAC TAC ACA ATG ATT GTT GGC GAT GAA CTC ACC ATC   1446
Pro Ile Glu Ser Asn Tyr Thr Met Ile Val Gly Asp Glu Leu Thr Ile
        375                 380                 385

ATG GAA GTG ACT GAA AGA GAT GCA GGA AAC TAC ACG GTC ATC CTC ACC   1494
Met Glu Val Thr Glu Arg Asp Ala Gly Asn Tyr Thr Val Ile Leu Thr
    390                 395                 400

AAC CCC ATT TCA ATG GAG AAA CAG AGC CAC ATG GTC TCT CTG GTT GTG   1542
Asn Pro Ile Ser Met Glu Lys Gln Ser His Met Val Ser Leu Val Val
    405                 410                 415

AAT GTC CCA CCC CAG ATC GGT GAG AAA GCC TTG ATC TCG CCT ATG GAT   1590
Asn Val Pro Pro Gln Ile Gly Glu Lys Ala Leu Ile Ser Pro Met Asp
420                 425                 430                 435

TCC TAC CAG TAT GGG ACC ATG CAG ACA TTG ACA TGC ACA GTC TAC GCC   1638
Ser Tyr Gln Tyr Gly Thr Met Gln Thr Leu Thr Cys Thr Val Tyr Ala
            440                 445                 450

AAC CCT CCC CTG CAC CAC ATC CAG TGG TAC TGG CAG CTA GAA GAA GCC   1686
Asn Pro Pro Leu His His Ile Gln Trp Tyr Trp Gln Leu Glu Glu Ala
        455                 460                 465

TGC TCC TAC AGA CCC GGC CAA ACA AGC CCG TAT GCT TGT AAA GAA TGG   1734
Cys Ser Tyr Arg Pro Gly Gln Thr Ser Pro Tyr Ala Cys Lys Glu Trp
    470                 475                 480

AGA CAC GTG GAG GAT TTC CAG GGG GGA AAC AAG ATC GAA GTC ACC AAA   1782
Arg His Val Glu Asp Phe Gln Gly Gly Asn Lys Ile Glu Val Thr Lys
    485                 490                 495

AAC CAA TAT GCC CTG ATT GAA GGA AAA AAC AAA ACT GTA AGT ACG CTG   1830
Asn Gln Tyr Ala Leu Ile Glu Gly Lys Asn Lys Thr Val Ser Thr Leu
500                 505                 510                 515

GTC ATC CAA GCT GCC AAC GTG TCA GCG TTG TAC AAA TGT GAA GCC ATC   1878
Val Ile Gln Ala Ala Asn Val Ser Ala Leu Tyr Lys Cys Glu Ala Ile
            520                 525                 530
```

AAC AAA GCG GGA CGA GGA GAG AGG GTC ATC TCC TTC CAT GTG ATC AGG 1926
Asn Lys Ala Gly Arg Gly Glu Arg Val Ile Ser Phe His Val Ile Arg
535 540 545

GGT CCT GAA ATT ACT GTG CAA CCT GCT GCC CAG CCA ACT GAG CAG GAG 1974
Gly Pro Glu Ile Thr Val Gln Pro Ala Ala Gln Pro Thr Glu Gln Glu
550 555 560

AGT GTG TCC CTG TTG TGC ACT GCA GAC AGA AAT ACG TTT GAG AAC CTC 2022
Ser Val Ser Leu Leu Cys Thr Ala Asp Arg Asn Thr Phe Glu Asn Leu
565 570 575

ACG TGG TAC AAG CTT GGC TCA CAG GCA ACA TCG GTC CAC ATG GGC GAA 2070
Thr Trp Tyr Lys Leu Gly Ser Gln Ala Thr Ser Val His Met Gly Glu
580 585 590 595

TCA CTC ACA CCA GTT TGC AAG AAC TTG GAT GCT CTT TGG AAA CTG AAT 2118
Ser Leu Thr Pro Val Cys Lys Asn Leu Asp Ala Leu Trp Lys Leu Asn
600 605 610

GGC ACC ATG TTT TCT AAC AGC ACA AAT GAC ATC TTG ATT GTG GCA TTT 2166
Gly Thr Met Phe Ser Asn Ser Thr Asn Asp Ile Leu Ile Val Ala Phe
615 620 625

CAG AAT GCC TCT CTG CAG GAC CAA GGC GAC TAT GTT TGC TCT GCT CAA 2214
Gln Asn Ala Ser Leu Gln Asp Gln Gly Asp Tyr Val Cys Ser Ala Gln
630 635 640

GAT AAG AAG ACC AAG AAA AGA CAT TGC CTG GTC AAA CAG CTC ATC ATC 2262
Asp Lys Lys Thr Lys Lys Arg His Cys Leu Val Lys Gln Leu Ile Ile
645 650 655

CTA GAG CGC ATG GCA CCC ATG ATC ACC GGA AAT CTG GAG AAT CAG ACA 2310
Leu Glu Arg Met Ala Pro Met Ile Thr Gly Asn Leu Glu Asn Gln Thr
660 665 670 675

ACA ACC ATT GGC GAG ACC ATT GAA GTG ACT TGC CCA GCA TCT GGA AAT 2358
Thr Thr Ile Gly Glu Thr Ile Glu Val Thr Cys Pro Ala Ser Gly Asn
680 685 690

CCT ACC CCA CAC ATT ACA TGG TTC AAA GAC AAC GAG ACC CTG GTA GAA 2406
Pro Thr Pro His Ile Thr Trp Phe Lys Asp Asn Glu Thr Leu Val Glu
695 700 705

GAT TCA GGC ATT GTA CTG AGA GAT GGG AAC CGG AAC CTG ACT ATC CGC 2454
Asp Ser Gly Ile Val Leu Arg Asp Gly Asn Arg Asn Leu Thr Ile Arg
710 715 720

AGG GTG AGG AAG GAG GAT GGA GGC CTC TAC ACC TGC CAG GCC TGC AAT 2502
Arg Val Arg Lys Glu Asp Gly Gly Leu Tyr Thr Cys Gln Ala Cys Asn
725 730 735

GTC CTT GGC TGT GCA AGA GCG GAG ACG CTC TTC ATA ATA GAA GGT GCC 2550
Val Leu Gly Cys Ala Arg Ala Glu Thr Leu Phe Ile Ile Glu Gly Ala
740 745 750 755

CAG GAA AAG ACC AAC TTG GAA GTC ATT ATC CTC GTC GGC ACT GCA GTG 2598
Gln Glu Lys Thr Asn Leu Glu Val Ile Ile Leu Val Gly Thr Ala Val
760 765 770

ATT GCC ATG TTC TTC TGG CTC CTT CTT GTC ATT GTC CTA CGG ACC GTT 2646
Ile Ala Met Phe Phe Trp Leu Leu Leu Val Ile Val Leu Arg Thr Val
775 780 785

AAG CGG GCC AAT GAA GGG GAA CTG AAG ACA GGC TAC TTG TCT ATT GTC 2694
Lys Arg Ala Asn Glu Gly Glu Leu Lys Thr Gly Tyr Leu Ser Ile Val
790 795 800

ATG GAT CCA GAT GAA TTG CCC TTG GAT GAG CGC TGT GAA CGC TTG CCT 2742
Met Asp Pro Asp Glu Leu Pro Leu Asp Glu Arg Cys Glu Arg Leu Pro
805 810 815

TAT GAT GCC AGC AAG TGG GAA TTC CCC AGG GAC CGG CTG AAA CTA GGA 2790
Tyr Asp Ala Ser Lys Trp Glu Phe Pro Arg Asp Arg Leu Lys Leu Gly
820 825 830 835

AAA CCT CTT GGC CGC GGT GCC TTC GGC CAA GTG ATT GAG GCA GAC GCT 2838
Lys Pro Leu Gly Arg Gly Ala Phe Gly Gln Val Ile Glu Ala Asp Ala
840 845 850

TTT GGA ATT GAC AAG ACA GCG ACT TGC AAA ACA GTA GCC GTC AAG ATG 2886
Phe Gly Ile Asp Lys Thr Ala Thr Cys Lys Thr Val Ala Val Lys Met
855 860 865

TTG AAA GAA GGA GCA ACA CAC AGC GAG CAT CGA GCC CTC ATG TCT GAA 2934
Leu Lys Glu Gly Ala Thr His Ser Glu His Arg Ala Leu Met Ser Glu
870 875 880

CTC AAG ATC CTC ATC CAC ATT GGT CAC CAT CTC AAT GTG GTG AAC CTC 2982
Leu Lys Ile Leu Ile His Ile Gly His His Leu Asn Val Val Asn Leu
885 890 895

CTA GGC GCC TGC ACC AAG CCG GGA GGG CCT CTC ATG GTG ATT GTG GAA 3030
Leu Gly Ala Cys Thr Lys Pro Gly Gly Pro Leu Met Val Ile Val Glu
900 905 910 915

TTC TGC AAG TTT GGA AAC CTA TCA ACT TAC TTA CGG GGC AAG ACA AAT 3078
Phe Cys Lys Phe Gly Asn Leu Ser Thr Tyr Leu Arg Gly Lys Arg Asn
920 925 930

GAA TTT GTT CCC TAT AAG AGC AAA GGG GCA CGC TTC CGC CAG GGC AAG 3126
Glu Phe Val Pro Tyr Lys Ser Lys Gly Ala Arg Phe Arg Gln Gly Lys
935 940 945

GAC TAC GTT GGG GAG CTC TCC GTG GAT CTG AAA AGA CGC TTG GAC AGC 3174
Asp Tyr Val Gly Glu Leu Ser Val Asp Leu Lys Arg Arg Leu Asp Ser
950 955 960

ATC ACC AGC AGC CAG AGC TCT GCC AGC TCA GGC TTT GTT GAG GAG AAA 3222
Ile Thr Ser Ser Gln Ser Ser Ala Ser Ser Gly Phe Val Glu Glu Lys
965 970 975

TCG CTC AGT GAT GTA GAG GAA GAA GAA GCT TCT GAA GAA CTG TAC AAG 3270
Ser Leu Ser Asp Val Glu Glu Glu Glu Ala Ser Glu Glu Leu Tyr Lys
980 985 990 995

GAC TTC CTG ACC TTG GAG CAT CTC ATC TGT TAC AGC TTC CAA GTG GCT 3318
Asp Phe Leu Thr Leu Glu His Leu Ile Cys Tyr Ser Phe Gln Val Ala
1000 1005 1010

AAG GGC ATG GAG TTC TTG GCA TCA AGG AAG TGT ATC CAC AGG GAC CTG 3366
Lys Gly Met Glu Phe Leu Ala Ser Arg Lys Cys Ile His Arg Asp Leu
1015 1020 1025

GCA GCA CGA AAC ATT CTC CTA TCG GAG AAG AAT GTG GTT AAG ATC TGT 3414
Ala Ala Arg Asn Ile Leu Leu Ser Glu Lys Asn Val Val Lys Ile Cys
1030 1035 1040

GAC TTC GGC TTG GCC CGG GAC ATT TAT AAA GAC CCG GAT TAT GTC AGA 3462
Asp Phe Gly Leu Ala Arg Asp Ile Tyr Lys Asp Pro Asp Tyr Val Arg
1045 1050 1055

AAA GGA GAT GCC CGA CTC CCT TTG AAG TGG ATG GCC CCG GAA ACC ATT 3510
Lys Gly Asp Ala Arg Leu Pro Leu Lys Trp Met Ala Pro Glu Thr Ile
1060 1065 1070 1075

TTT GAC AGA GTA TAC ACA ATT CAG AGC GAT GTG TGG TCT TTC GGT GTG 3558
Phe Asp Arg Val Tyr Thr Ile Gln Ser Asp Val Trp Ser Phe Gly Val
1080 1085 1090

TTG CTC TGG GAA ATA TTT TCC TTA GGT GCC TCC CCA TAC CCT GGG GTC 3606
Leu Leu Trp Glu Ile Phe Ser Leu Gly Ala Ser Pro Tyr Pro Gly Val
1095 1100 1105

AAG ATT GAT GAA GAA TTT TGT AGG AGA TTG AAA GAA GGA ACT AGA ATG 3654
Lys Ile Asp Glu Glu Phe Cys Arg Arg Leu Lys Glu Gly Thr Arg Met
1110 1115 1120

CGG GCT CCT GAC TAC ACT ACC CCA GAA ATG TAC CAG ACC ATG CTG GAC 3702
Arg Ala Pro Asp Tyr Thr Thr Pro Glu Met Tyr Gln Thr Met Leu Asp
1125 1130 1135

TGC TGG CAT GAG GAC CCC AAC CAG AGA CCC TCG TTT TCA GAG TTG GTG 3750
Cys Trp His Glu Asp Pro Asn Gln Arg Pro Ser Phe Ser Glu Leu Val
1140 1145 1150 1155

GAG CAT TTG GGA AAC CTC CTG CAA GCA AAT GCG CAG CAG GAT GGC AAA 3798
Glu His Leu Gly Asn Leu Leu Gln Ala Asn Ala Gln Gln Asp Gly Lys
1160 1165 1170

GAC TAT ATT GTT CTT CCA ATG TCA GAG ACA CTG AGC ATG GAA GAG GAT 3846
Asp Tyr Ile Val Leu Pro Met Ser Glu Thr Leu Ser Met Glu Glu Asp
1175 1180 1185

TCT GGA CTC TCC CTG CCT ACC TCA CCT GTT TCC TGT ATG GAG GAA GAG 3894
Ser Gly Leu Ser Leu Pro Thr Ser Pro Val Ser Cys Met Glu Glu Glu
1190 1195 1200

GAA GTG TGC GAC CCC AAA TTC CAT TAT GAC AAC ACA GCA GGA ATC AGT 3942
Glu Val Cys Asp Pro Lys Phe His Tyr Asp Asn Thr Ala Gly Ile Ser
1205 1210 1215

CAT TAT CTC CAG AAC AGT AAG CGA AAG AGC CGG CCA GTG AGT GTA AAA 3990
His Tyr Leu Gln Asn Ser Lys Arg Lys Ser Arg Pro Val Ser Val Lys
1220 1225 1230 1235

ACA TTT GAA GAT ATC CCA TTG GAG GAA CCA GAA GTA AAA GTG ATC CCA 4038
Thr Phe Glu Asp Ile Pro Leu Glu Glu Pro Glu Val Lys Val Ile Pro
1240 1245 1250

GAT GAC AGC CAG ACA GAC AGT GGG ATG GTC CTT GCA TCA GAA GAG CTG 4086
Asp Asp Ser Gln Thr Asp Ser Gly Met Val Leu Ala Ser Glu Glu Leu
1255 1260 1265

AAA ACT CTG GAA GAC AGG AAC AAA TTA TCT CCA TCT TTT GGT GGA ATG 4134
Lys Thr Leu Glu Asp Arg Asn Lys Leu Ser Pro Ser Phe Gly Gly Met
1270 1275 1280

ATG CCC AGT AAA AGC AGG GAG TCT GTG GCC TCG GAA GGC TCC AAC CAG 4182
Met Pro Ser Lys Ser Arg Glu Ser Val Ala Ser Glu Gly Ser Asn Gln
1285 1290 1295

ACC AGT GGC TAC CAG TCT GGG TAT CAC TCA GAT GAC ACA GAC ACC ACC 4230
Thr Ser Gly Tyr Gln Ser Gly Tyr His Ser Asp Asp Thr Asp Thr Thr
1300 1305 1310 1315

GTG TAC TCC AGC GAC GAG GCA GGA CTT TTA AAG ATG GTG GAT GCT GCA 4278
Val Tyr Ser Ser Asp Glu Ala Gly Leu Leu Lys Met Val Asp Ala Ala
1320 1325 1330

GTT CAC GCT GAC TCA GGG ACC ACA CTG CAG CTC ACC TCC TGT TTA AAT 4326
Val His Ala Asp Ser Gly Thr Thr Leu Gln Leu Thr Ser Cys Leu Asn
1335 1340 1345

GGA AGT GGT CCT GTC CCG GCT CCG CCC CCA ACT CCT GGA AAT CAC GAG 4374
Gly Ser Gly Pro Val Pro Ala Pro Pro Pro Thr Pro Gly Asn His Glu
1350 1355 1360

AGA GGT GCT GCT TAGATTTTCA AGTGTTGTTC TTTCCACCAC CCGGAAGTAG 4426
Arg Gly Ala Ala
1365

CCACATTTGA TTTTCATTTT TGGAGGAGGG ACCTCAGACT GCAAGGAGCT TGTCCTCAGG 4486

GCATTTCCAG AGAAGATGCC CATGACCCAA GAATGTGTTG ACTCTACTCT CTTTTCCATT 4546

CATTTAAAAG TCCTATATAA TGTGCCCTGC TGTGGTCTCA CTACCAGTTA AAGCAAAAGA 4606

CTTTCAAACA CGTGGACTCT GTCCTCCAAG AAGTGGCAAC GGCACCTCTG TGAAACTGGA 4666

TCGAATGGGC AATGCTTTGT GTGTTGAGGA TGGGTGAGAT GTCCCAGGGC CGAGTCTGTC 4726

TACCTTGGAG GCTTTGTGGA GGATGCGGGC TATGAGCCAA GTGTTAAGTG TGGGATGTGG 4786

ACTGGGAGGA AGGAAGGCGC AAGTCGCTCG GAGAGCGGTT GGAGCCTGCA GATGCATTGT 4846

GCTGGCTCTG GTGGAGGTGG GCTTGTGGCC TGTCAGGAAA CGCAAAGGCG GCCGGCAGGG 4906

TTTGGTTTTG GAAGGTTTGC GTGCTCTTCA CAGTCGGGTT ACAGGCGAGT TCCCTGTGGC 4966

GTTTCCTACT CCTAATGAGA GTTCCTTCCG GACTCTTACG TGTCTCCTGG CCTGGCCCCA 5026

GGAAGGAAAT GATGCAGCTT GCTCCTTCCT CATCTCTCAG GCTGTGCCTT AATTCAGAAC 5086

ACCAAAAGAG AGGAACGTCG GCAGAGGCTC CTGACGGGGC CGAAGAATTG TGAGAACAGA 5146

ACAGAAACTC AGGGTTTCTG CTGGGTGGAG ACCCACGTGG CGCCCTGGTG GCAGGTCTGA 5206

GGGTTCTCTG TCAAGTGGCG GTAAAGGCTC AGGCTGGTGT TCTTCCTCTA TCTCCACTCC 5266

TGTCAGGCCC CCAAGTCCTC AGTATTTTAG CTTTGTGGCT TCCTGATGGC AGAAAAATCT 5326

TAATTGGTTG GTTTGCTCTC CAGATAATCA CTAGCCAGAT TTCGAAATTA CTTTTTAGCC 5386

GAGGTTATGA TAACATCTAC TGTATCCTTT AGAATTTTAA CCTATAAAAC TATGTCTACT 5446

GGTTTCTGCC TGTGTGCTTA TGTT 5470

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1367 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Glu Ser Lys Ala Leu Leu Ala Val Ala Leu Trp Phe Cys Val Glu
1 5 10 15

Thr Arg Ala Ala Ser Val Gly Leu Thr Gly Asp Phe Leu His Pro Pro
20 25 30

Lys Leu Ser Thr Gln Lys Asp Ile Leu Thr Ile Leu Ala Asn Thr Thr
35 40 45

Leu Gln Ile Thr Cys Arg Gly Gln Arg Asp Leu Asp Trp Leu Trp Pro
50 55 60

Asn Ala Gln Arg Asp Ser Glu Glu Arg Val Leu Val Thr Glu Cys Gly
65 70 75 80

Gly Gly Asp Ser Ile Phe Cys Lys Thr Leu Thr Ile Pro Arg Val Val
85 90 95

Gly Asn Asp Thr Gly Ala Tyr Lys Cys Ser Tyr Arg Asp Val Asp Ile
100 105 110

Ala Ser Thr Val Tyr Val Tyr Val Arg Asp Tyr Arg Ser Pro Phe Ile
115 120 125

Ala Ser Val Ser Asp Gln His Gly Ile Val Tyr Ile Thr Glu Asn Lys
130 135 140

Asn Lys Thr Val Val Ile Pro Cys Arg Gly Ser Ile Ser Asn Leu Asn
145 150 155 160

Val Ser Leu Cys Ala Arg Tyr Pro Glu Lys Arg Phe Val Pro Asp Gly
165 170 175

Asn Arg Ile Ser Trp Asp Ser Glu Ile Gly Phe Thr Leu Pro Ser Tyr
180 185 190

Met Ile Ser Tyr Ala Gly Met Val Phe Cys Glu Ala Lys Ile Asn Asp
195 200 205

Glu Thr Tyr Gln Ser Ile Met Tyr Ile Val Val Val Val Gly Tyr Arg
210 215 220

Ile Tyr Asp Val Ile Leu Ser Pro Pro His Glu Ile Glu Leu Ser Ala
225 230 235 240

Gly Glu Lys Leu Val Leu Asn Cys Thr Ala Arg Thr Glu Leu Asn Val
245 250 255

Gly Leu Asp Phe Thr Trp His Ser Pro Pro Ser Lys Ser His His Lys
260 265 270

Lys Ile Val Asn Arg Asp Val Lys Pro Phe Pro Gly Thr Val Ala Lys
275 280 285

Met Phe Leu Ser Thr Leu Thr Ile Glu Ser Val Thr Lys Ser Asp Gln
290 295 300

Gly Glu Tyr Thr Cys Val Ala Ser Ser Gly Arg Met Ile Lys Arg Asn
305 310 315 320

Arg Thr Phe Val Arg Val His Thr Lys Pro Phe Ile Ala Phe Gly Ser
325 330 335

Gly Met Lys Ser Leu Val Glu Ala Thr Val Gly Ser Gln Val Arg Ile
340 345 350

Pro Val Lys Tyr Leu Ser Tyr Pro Ala Pro Asp Ile Lys Trp Tyr Arg
355 360 365

Asn Gly Arg Pro Ile Glu Ser Asn Tyr Thr Met Ile Val Gly Asp Glu
370 375 380

Leu Thr Ile Met Glu Val Thr Glu Arg Asp Ala Gly Asn Tyr Thr Val
385 390 395 400

Ile Leu Thr Asn Pro Ile Ser Met Glu Lys Gln Ser His Met Val Ser
405 410 415

Leu Val Val Asn Val Pro Pro Gln Ile Gly Glu Lys Ala Leu Ile Ser

420 425 430

Pro Met Asp Ser Tyr Gln Tyr Gly Thr Met Gln Thr Leu Thr Cys Thr
435 440 445

Val Tyr Ala Asn Pro Pro Leu His His Ile Gln Trp Tyr Trp Gln Leu
450 455 460

Glu Glu Ala Cys Ser Tyr Arg Pro Gly Gln Thr Ser Pro Tyr Ala Cys
465 470 475 480

Lys Glu Trp Arg His Val Glu Asp Phe Gln Gly Gly Asn Lys Ile Glu
485 490 495

Val Thr Lys Asn Gln Tyr Ala Leu Ile Glu Gly Lys Asn Lys Thr Val
500 505 510

Ser Thr Leu Val Ile Gln Ala Ala Asn Val Ser Ala Leu Tyr Lys Cys
515 520 525

Glu Ala Ile Asn Lys Ala Gly Arg Gly Glu Arg Val Ile Ser Phe His
530 535 540

Val Ile Arg Gly Pro Glu Ile Thr Val Gln Pro Ala Ala Gln Pro Thr
545 550 555 560

Glu Gln Glu Ser Val Ser Leu Leu Cys Thr Ala Asp Arg Asn Thr Phe
565 570 575

Glu Asn Leu Thr Trp Tyr Lys Leu Gly Ser Gln Ala Thr Ser Val His
580 585 590

Met Gly Glu Ser Leu Thr Pro Val Cys Lys Asn Leu Asp Ala Leu Trp
595 600 605

Lys Leu Asn Gly Thr Met Phe Ser Asn Ser Thr Asn Asp Ile Leu Ile
610 615 620

Val Ala Phe Gln Asn Ala Ser Leu Gln Asp Gln Gly Asp Tyr Val Cys
625 630 635 640

Ser Ala Gln Asp Lys Lys Thr Lys Lys Arg His Cys Leu Val Lys Gln
645 650 655

Leu Ile Ile Leu Glu Arg Met Ala Pro Met Ile Thr Gly Asn Leu Glu
660 665 670

Asn Gln Thr Thr Thr Ile Gly Glu Thr Ile Glu Val Thr Cys Pro Ala
675 680 685

Ser Gly Asn Pro Thr Pro His Ile Thr Trp Phe Lys Asp Asn Glu Thr
690 695 700

Leu Val Glu Asp Ser Gly Ile Val Leu Arg Asp Gly Asn Arg Asn Leu
705 710 715 720

Thr Ile Arg Arg Val Arg Lys Glu Asp Gly Gly Leu Tyr Thr Cys Gln
725 730 735

Ala Cys Asn Val Leu Gly Cys Ala Arg Ala Glu Thr Leu Phe Ile Ile
740 745 750

Glu Gly Ala Gln Glu Lys Thr Asn Leu Glu Val Ile Ile Leu Val Gly
755 760 765

Thr Ala Val Ile Ala Met Phe Phe Trp Leu Leu Leu Val Ile Val Leu
770 775 780

Arg Thr Val Lys Arg Ala Asn Glu Gly Glu Leu Lys Thr Gly Tyr Leu
785 790 795 800

Ser Ile Val Met Asp Pro Asp Glu Leu Pro Leu Asp Glu Arg Cys Glu
805 810 815

Arg Leu Pro Tyr Asp Ala Ser Lys Trp Glu Phe Pro Arg Asp Arg Leu
820 825 830

Lys Leu Gly Lys Pro Leu Gly Arg Gly Ala Phe Gly Gln Val Ile Glu
835 840 845

Ala Asp Ala Phe Gly Ile Asp Lys Thr Ala Thr Cys Lys Thr Val Ala
850 855 860

Val Lys Met Leu Lys Glu Gly Ala Thr His Ser Glu His Arg Ala Leu
865 870 875 880

Met Ser Glu Leu Lys Ile Leu Ile His Ile Gly His His Leu Asn Val
885 890 895

Val Asn Leu Leu Gly Ala Cys Thr Lys Pro Gly Gly Pro Leu Met Val
900 905 910

Ile Val Glu Phe Cys Lys Phe Gly Asn Leu Ser Thr Tyr Leu Arg Gly
915 920 925

Lys Arg Asn Glu Phe Val Pro Tyr Lys Ser Lys Gly Ala Arg Phe Arg
930 935 940

Gln Gly Lys Asp Tyr Val Gly Glu Leu Ser Val Asp Leu Lys Arg Arg
945 950 955 960

Leu Asp Ser Ile Thr Ser Ser Gln Ser Ser Ala Ser Ser Gly Phe Val
965 970 975

Glu Glu Lys Ser Leu Ser Asp Val Glu Glu Glu Glu Ala Ser Glu Glu
980 985 990

Leu Tyr Lys Asp Phe Leu Thr Leu Glu His Leu Ile Cys Tyr Ser Phe
995 1000 1005

Gln Val Ala Lys Gly Met Glu Phe Leu Ala Ser Arg Lys Cys Ile His
1010 1015 1020

Arg Asp Leu Ala Ala Arg Asn Ile Leu Leu Ser Glu Lys Asn Val Val
1025 1030 1035 1040

Lys Ile Cys Asp Phe Gly Leu Ala Arg Asp Ile Tyr Lys Asp Pro Asp
1045 1050 1055

Tyr Val Arg Lys Gly Asp Ala Arg Leu Pro Leu Lys Trp Met Ala Pro
1060 1065 1070

Glu Thr Ile Phe Asp Arg Val Tyr Thr Ile Gln Ser Asp Val Trp Ser
1075 1080 1085

Phe Gly Val Leu Leu Trp Glu Ile Phe Ser Leu Gly Ala Ser Pro Tyr
1090 1095 1100

Pro Gly Val Lys Ile Asp Glu Glu Phe Cys Arg Arg Leu Lys Glu Gly
1105 1110 1115 1120

Thr Arg Met Arg Ala Pro Asp Tyr Thr Thr Pro Glu Met Tyr Gln Thr
1125 1130 1135

Met Leu Asp Cys Trp His Glu Asp Pro Asn Gln Arg Pro Ser Phe Ser

1140 1145 1150

Glu Leu Val Glu His Leu Gly Asn Leu Leu Gln Ala Asn Ala Gln Gln
1155 1160 1165

Asp Gly Lys Asp Tyr Ile Val Leu Pro Met Ser Glu Thr Leu Ser Met
1170 1175 1180

Glu Glu Asp Ser Gly Leu Ser Leu Pro Thr Ser Pro Val Ser Cys Met
1185 1190 1195 1200

Glu Glu Glu Glu Val Cys Asp Pro Lys Phe His Tyr Asp Asn Thr Ala
1205 1210 1215

Gly Ile Ser His Tyr Leu Gln Asn Ser Lys Arg Lys Ser Arg Pro Val
1220 1225 1230

Ser Val Lys Thr Phe Glu Asp Ile Pro Leu Glu Glu Pro Glu Val Lys
1235 1240 1245

Val Ile Pro Asp Asp Ser Gln Thr Asp Ser Gly Met Val Leu Ala Ser
1250 1255 1260

Glu Glu Leu Lys Thr Leu Glu Asp Arg Asn Lys Leu Ser Pro Ser Phe
1265 1270 1275 1280

Gly Gly Met Met Pro Ser Lys Ser Arg Glu Ser Val Ala Ser Glu Gly
1285 1290 1295

Ser Asn Gln Thr Ser Gly Tyr Gln Ser Gly Tyr His Ser Asp Asp Thr
1300 1305 1310

Asp Thr Thr Val Tyr Ser Ser Asp Glu Ala Gly Leu Leu Lys Met Val
1315 1320 1325

Asp Ala Ala Val His Ala Asp Ser Gly Thr Thr Leu Gln Leu Thr Ser
1330 1335 1340

Cys Leu Asn Gly Ser Gly Pro Val Pro Ala Pro Pro Pro Thr Pro Gly
1345 1350 1355 1360

Asn His Glu Arg Gly Ala Ala
1365

WHAT IS CLAIMED IS:

1. A recombinant DNA vector containing a nucleotide sequence that encodes a Flk-1 operatively associated with a regulatory sequence that controls gene expression in a host.

2. A recombinant DNA vector containing a nucleotide sequence that encodes a Flk-1 fusion protein operatively associated with a regulatory sequence that controls gene expression in a host.

3. An engineered host cell that contains the recombinant DNA vector of Claims 1 or 2.

4. An engineered cell line that contains the recombinant DNA expression vector of Claim 1 and expresses Flk-1.

5. The engineered cell line of Claim 3 which expresses the Flk-1 on the surface of the cell.

6. An engineered cell line that contains the recombinant DNA expression vector of Claim 2 and expresses the Flk-1 fusion protein.

7. The engineered cell line of Claim 6 that expresses the Flk-1 fusion protein on the surface of the cell.

8. A method for producing recombinant Flk-1, comprising:

(a) culturing a host cell transformed with the recombinant DNA expression vector of Claim 1 and which expresses the Flk-1; and

(b) recovering the Flk-1 gene product from the cell culture.

9. A method for producing recombinant Flk-1 fusion protein, comprising:

(a) culturing a host cell transformed with the recombinant DNA expression vector of Claim 2 and which expresses the Flk-1 fusion protein; and

(b) recovering the Flk-1 fusion protein from the cell culture.

10. An isolated recombinant Flk-1 receptor protein.

11. A fusion protein comprising Flk-1 linked to a heterologous protein or peptide sequence.

12. An oligonucleotide which encodes an antisense sequence complementary to a portion of the Flk-1 nucleotide sequence, and which inhibits translation of the Flk-1 gene in a cell.

13. The oligonucleotide of Claim 12 which is complementary to a nucleotide sequence encoding the amino terminal region of the Flk-1.

14. A monoclonal antibody which immunospecifically binds to an epitope of the Flk-1.

15. The monoclonal antibody of Claim 14 which competitively inhibits the binding of VEGF to the Flk-1.

16. The monoclonal antibody of Claim 14 which is linked to a cytotoxic agent.

17. The monoclonal antibody of Claim 14 which is linked to a radioisotope.

18. A method for screening and identifying antagonists of VEGF, comprising:

(a) contacting a cell line that expresses Flk-1 with a test compound in the presence of VEGF; and

(b) determining whether the test compound inhibits the binding and cellular effects of VEGF on the cell line,

in which antagonists are identified as those compounds that inhibit both the binding and cellular effects of VEGF on the cell line.

19. A method for screening and identifying agonists of VEGF, comprising:

(a) contacting a cell line that expresses the Flk-1 with a test compound in the presence and in the absence of VEGF;

(b) determining whether, in the presence of VEGF, the test compound inhibits the binding of VEGF to the cell line; and

(c) determining whether, in the absence of the VEGF, the test compound mimics the cellular effects of VEGF on the cell line,

in which agonists are identified as those test compounds that inhibit the binding but mimic the cellular effects of VEGF on the cell line.

20. The method according to Claims 18 or 19 in which the cell line is a genetically engineered cell line.

21. The method according to Claims 18 or 19 in which the cell line endogenously expresses the Flk-1.

22. A method for screening and identifying antagonists of VEGF comprising:

(a) contacting Flk-1 protein with a random peptide library such that Flk-1 will recognize and bind to one or more peptide species within the library;
(b) isolating the Flk-1/peptide combination;
(c) determining the sequence of the peptide isolated in step c; and
(d) determining whether the test compound inhibits the binding and cellular effects of VEGF,

in which antagonists are identified as those peptides that inhibit both the binding and cellular effects of VEGF.

23. A method for screening and identifying agonists of VEGF comprising:

(a) contacting Flk-1 protein with a random peptide library such that Flk-1 will recognize and bind to one or more peptide species within the library;
(b) isolating the Flk-1/peptide combination;
(c) determining the sequence of the peptide isolated in step c; and
(d) determining whether, in the absence of the VRGF, the peptide mimics the cellular effects of VEGF,

in which agonists are identified as those peptides that inhibit the binding but mimic the cellular effects of Flk-1.

24. The method according to Claims 22 or 23 in which the Flk-1 protein is genetically engineered.

25. A method of modulating the endogenous enzymatic activity of the tyrosine kinase Flk-1 receptor in a mammal comprising administering to the mammal an effective amount of a ligand to the Flk-1 receptor protein to modulate the enzymatic activity.

26. The method of Claim 25 in which the ligand to the Flk-1 receptor is VEGF.

27. The method of Claim 25 in which the ligand to the Flk-1 receptor is a VEGF agonist.

28. The method of Claim 25 in which the ligand to the Flk-1 receptor is an antagonist of VEGF.

29. The antagonist of Claim 28 that is a monoclonal antibody which immunospecifically binds to an epitope of Flk-1.

30. The antagonist of Claim 28 that is a soluble Flk-1 receptor.

31. The method of Claim 25 in which the enzymatic activity of the receptor protein is increased.

32. The method of Claim 25 in which the enzymatic activity of the receptor protein is decreased.

33. The method of Claim 31 in which the ligand stimulates endothelial cell proliferation.

34. The method of Claim 32 in which the ligand inhibits endothelial cell proliferation.

35. The method of Claim 32 in which the ligand inhibits angiogenesis.

36. A recombinant vector containing a nucleotide sequence that encodes a truncated Flk-1 which has dominant-negative activity which inhibits the cellular effects of VEGF binding.

37. The recombinant vector of claim 36 containing a nucleotide sequence encoding amino acids 1 through 806 of Flk-1.

38. The recombinant vector of claim 36 in which the vector is a retrovirus vector.

39. The recombinant vector of claim 38 containing a nucleotide sequence encoding amino acids 1 through 806 of Flk-1.

40. An engineered cell line that contains the recombinant DNA vector of Claim 36 and expresses truncated Flk-1.

41. An engineered cell line that contains the recombinant vector of Claim 38 or 39 and produces infectious retrovirus particles expressing truncated Flk-1.

42. An isolated recombinant truncated Flk-1 receptor protein which has dominant-negative activity which inhibits the cellular effects of VEGF binding.

43. A method of modulating the cellular effects of VEGF in a mammal comprising administrating to the mammal an effective amount of truncated Flk-1 receptor protein which inhibits the cellular effects of VEGF binding.

```
FLK-1  866  ILIHIGHHLNVVNLLGACTKPGGPLMVIVEFSKFGNLSTYLRGKRNEFVPYKSKGARFRQ
KDR         -------------------------------C-D--------S---------T-------
TKR-C       -------------------------------C---------------------------S

FLK-1  926  GKDYVGELSVDLKRRLDSITSSQSSASSGFVEEKSLSDVEEEEASEELYKDFLTLEHLIC
KDR         ------AIP-----------------------------------P-D-------------
TKR-C       ------------------------------------------------------------

FLK-1  986  YSFQVAKGMEFLASRKCIHRDLAARNILLSEKNVVKICDFGLARDIYKDPDYVRKGDARL
KDR         ------------------------------------------------------------
TKR-C       ------------------------------------------------------------
```

FIG.1

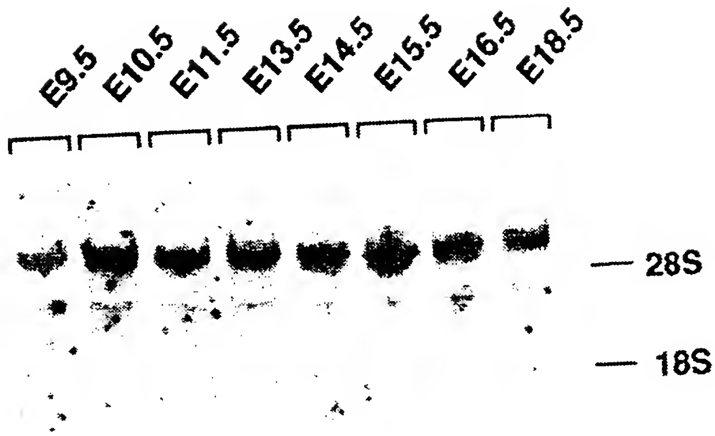
E9.5
E10.5
E11.5
E13.5
E14.5
E15.5
E16.5
E18.5
28S
18S

FIG. 2A

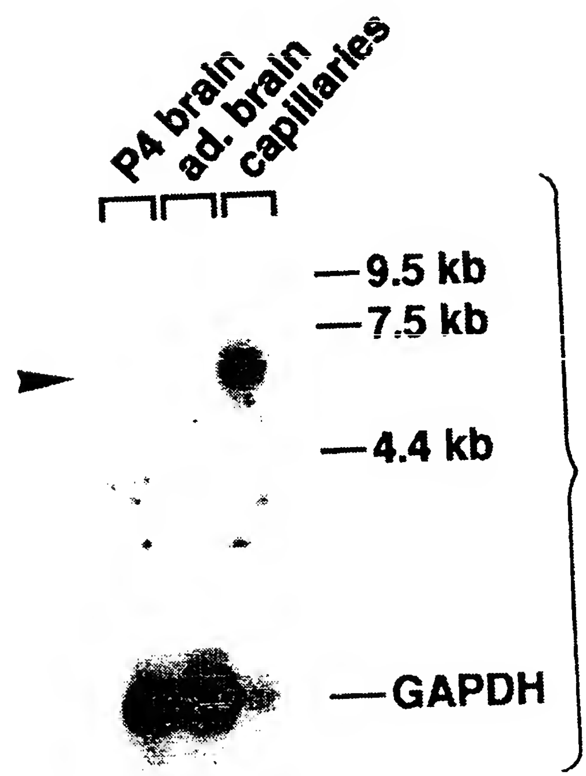
P4 brain
ad. brain
capillaries
9.5 kb
7.5 kb
4.4 kb
GAPDH

FIG. 2B

FIG. 3A

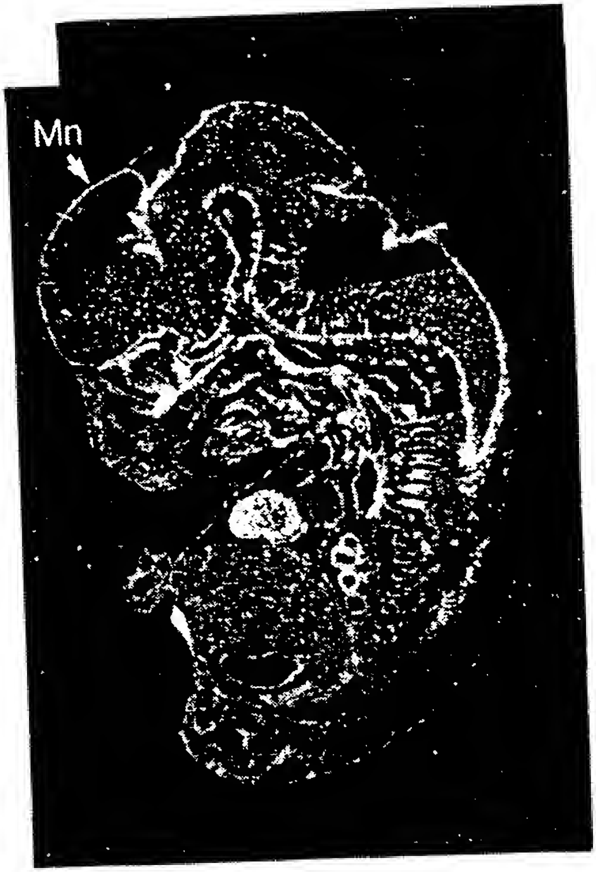

FIG. 3B

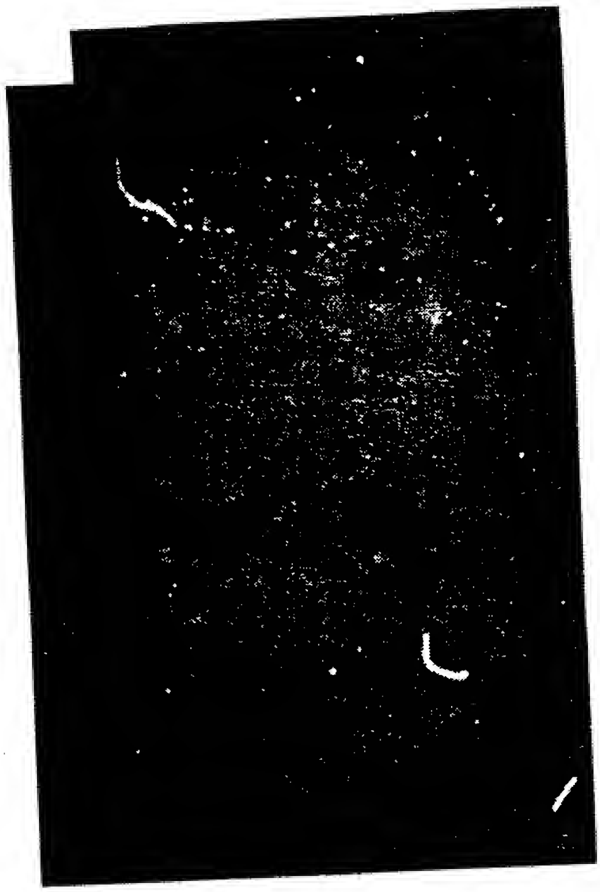

FIG. 3C

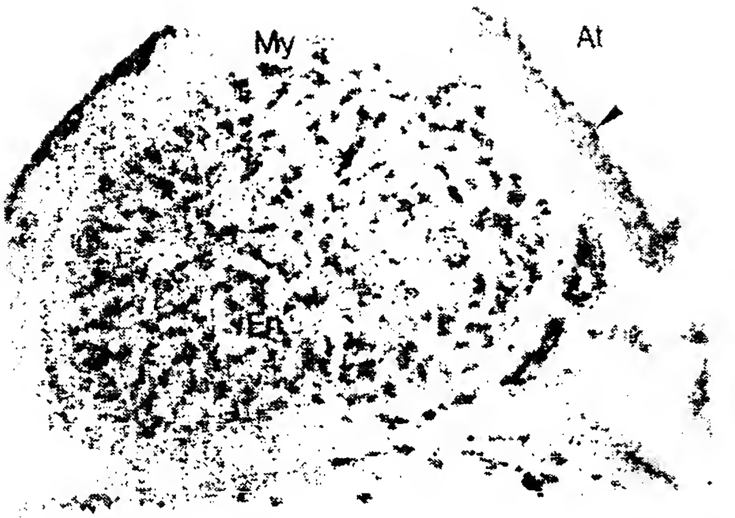

FIG. 4A

FIG. 4B

FIG. 4D

FIG. 4C

FIG. 4E

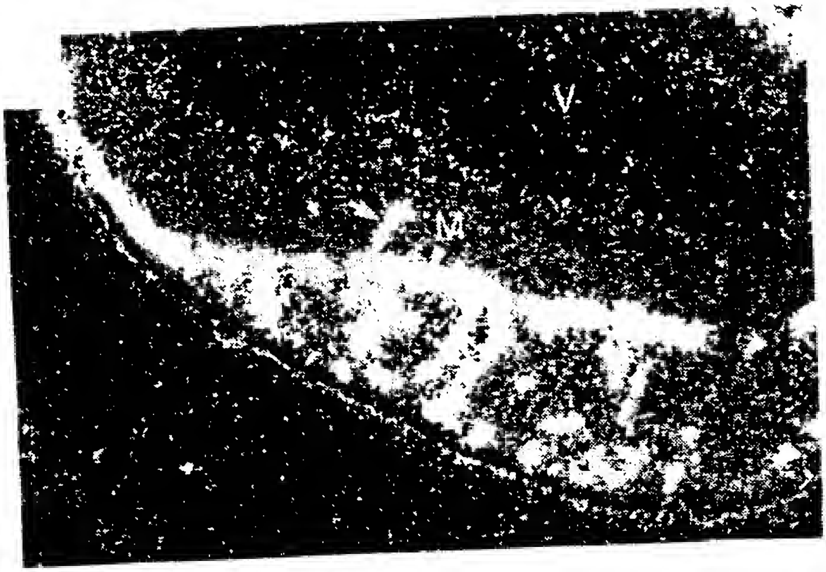

FIG. 5A

FIG. 5B

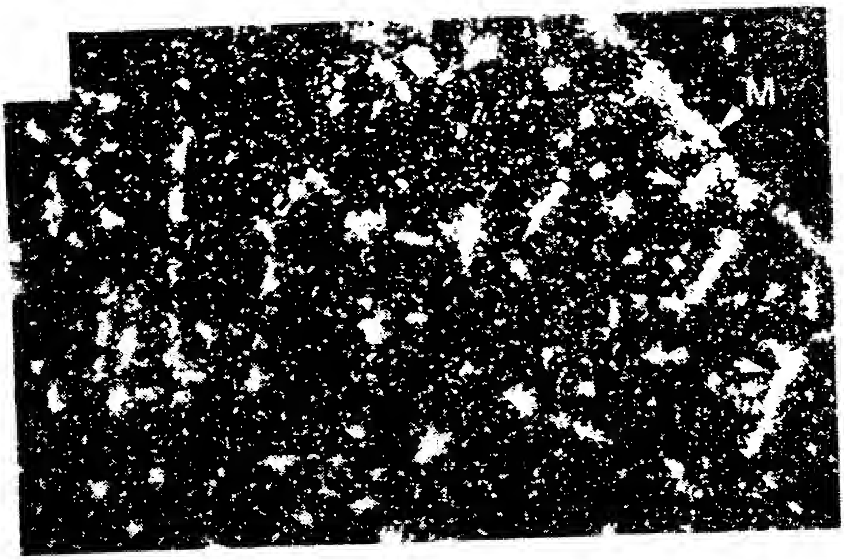

FIG. 5C

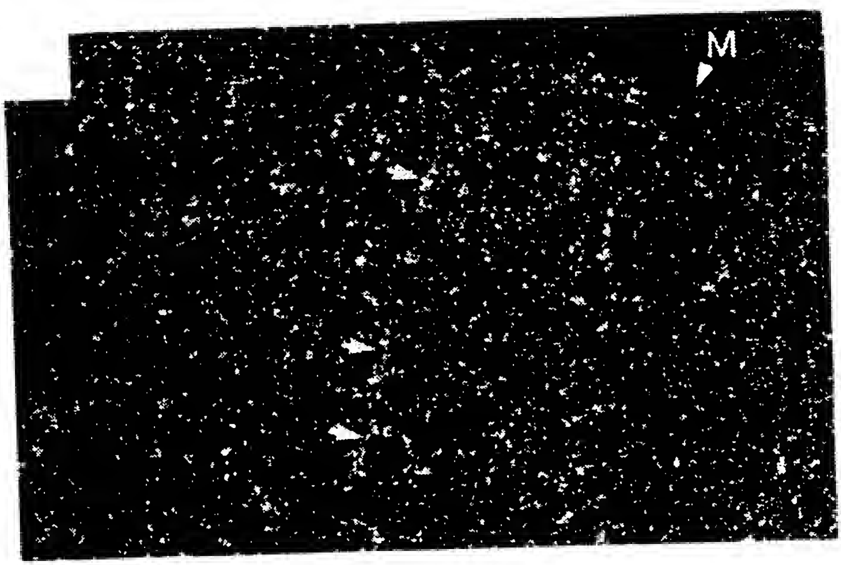

FIG. 5D

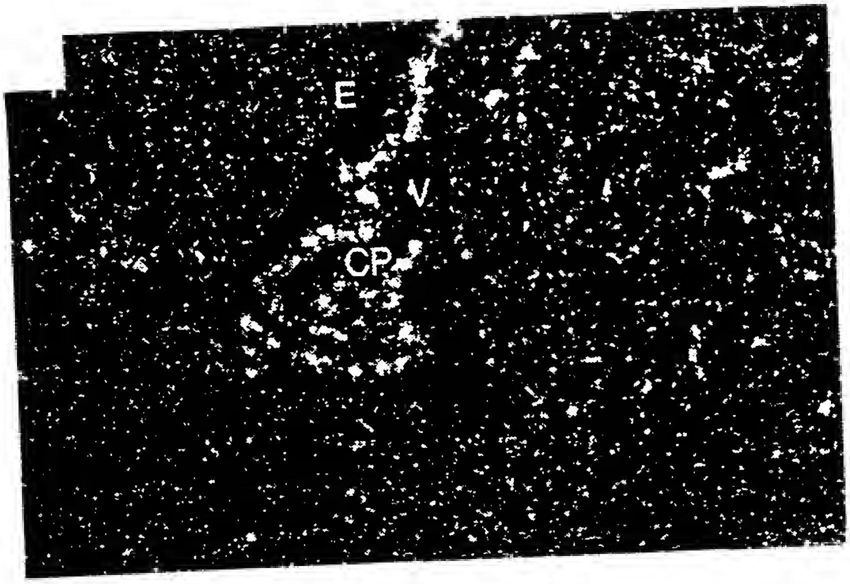

FIG. 6A

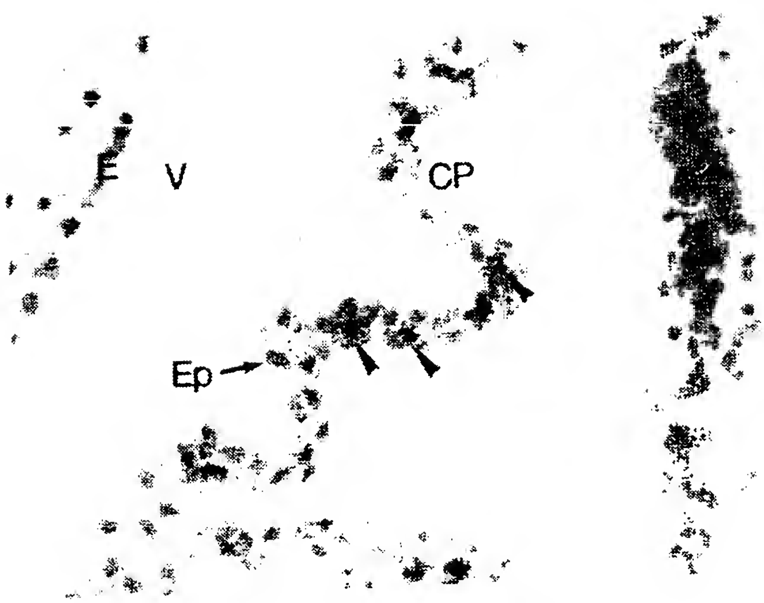

FIG. 6B

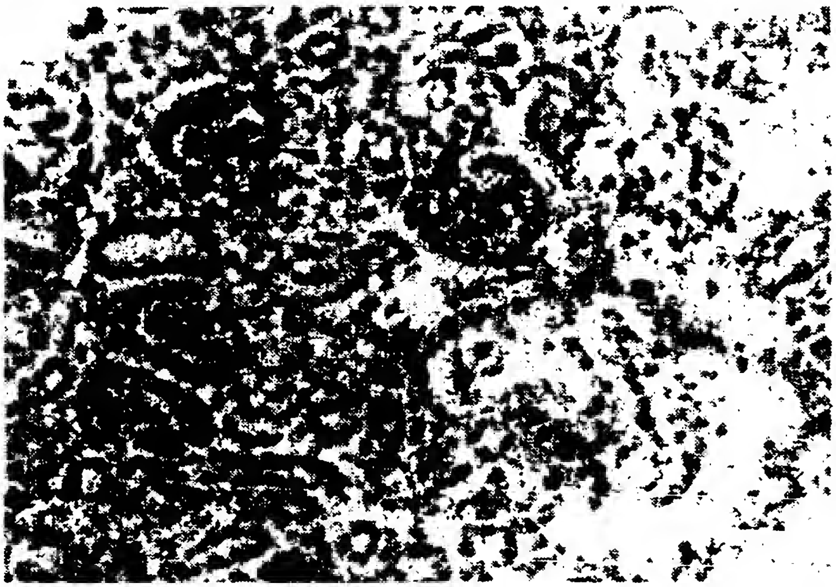

FIG. 7A

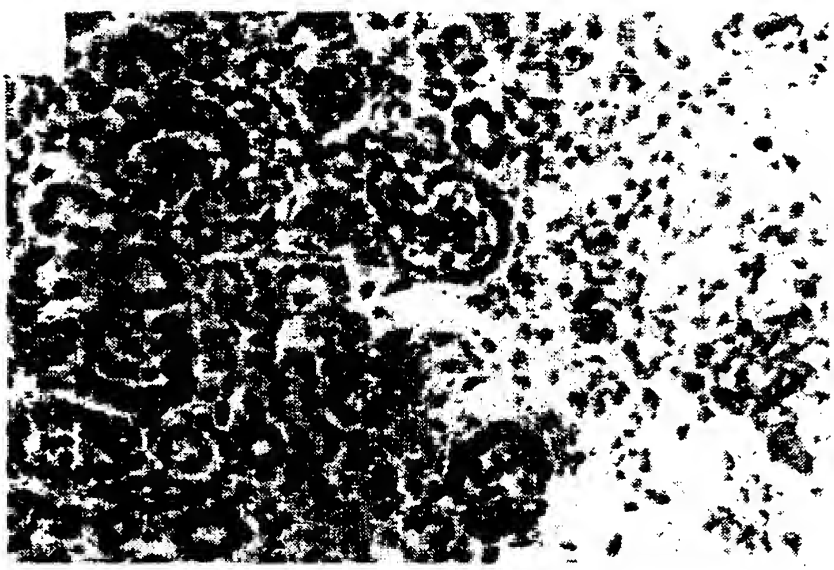

FIG. 7B

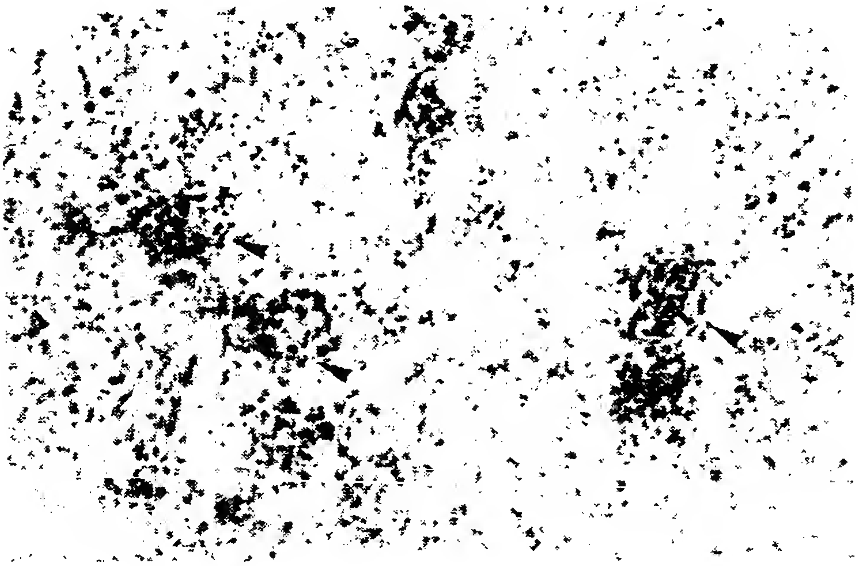

FIG. 7C

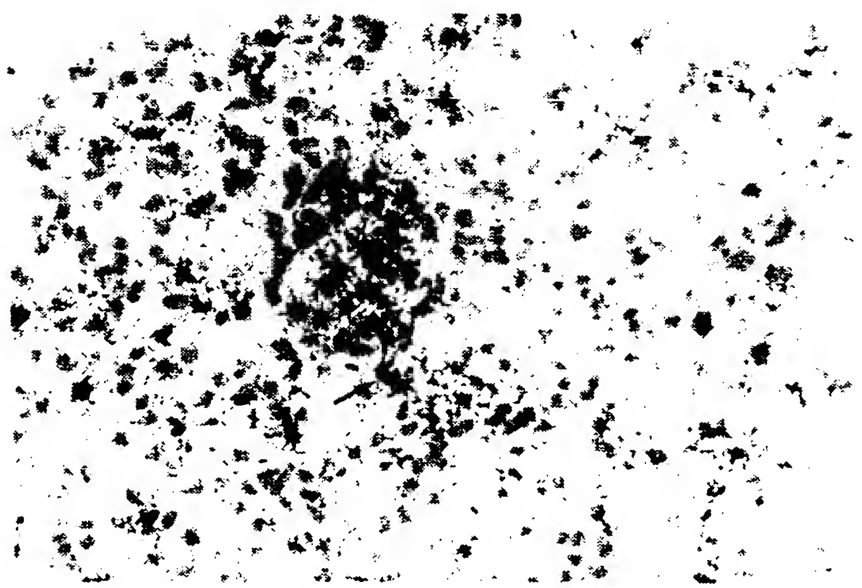

FIG. 7D

FIG. 8B

FIG. 8A

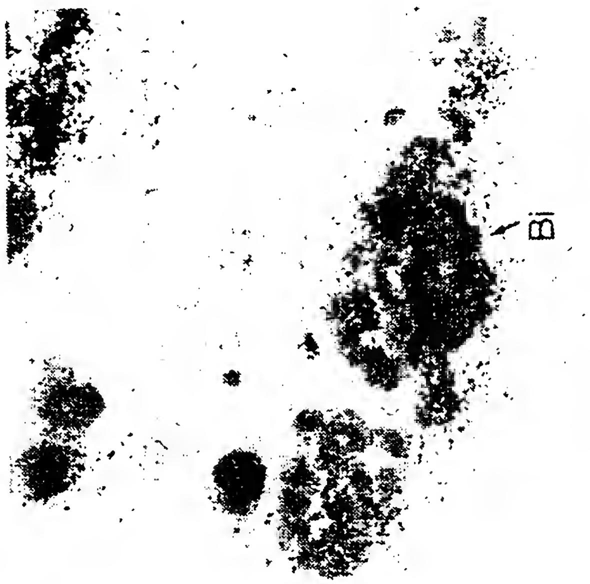

FIG. 8D

FIG. 8C

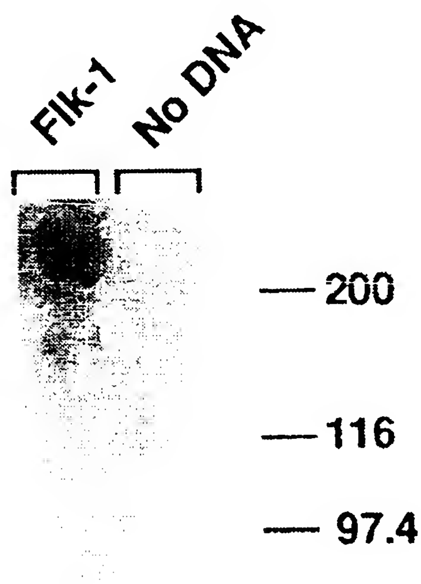

FIG. 9A

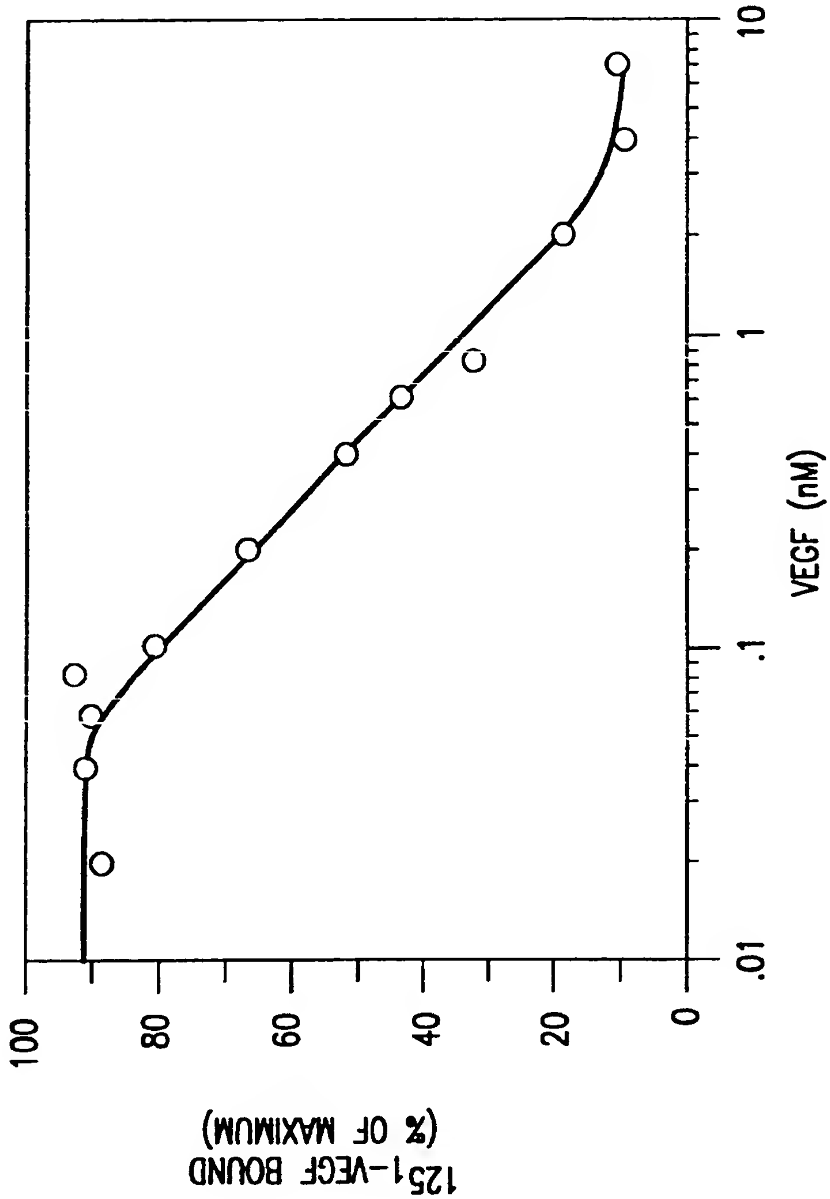

FIG.9B

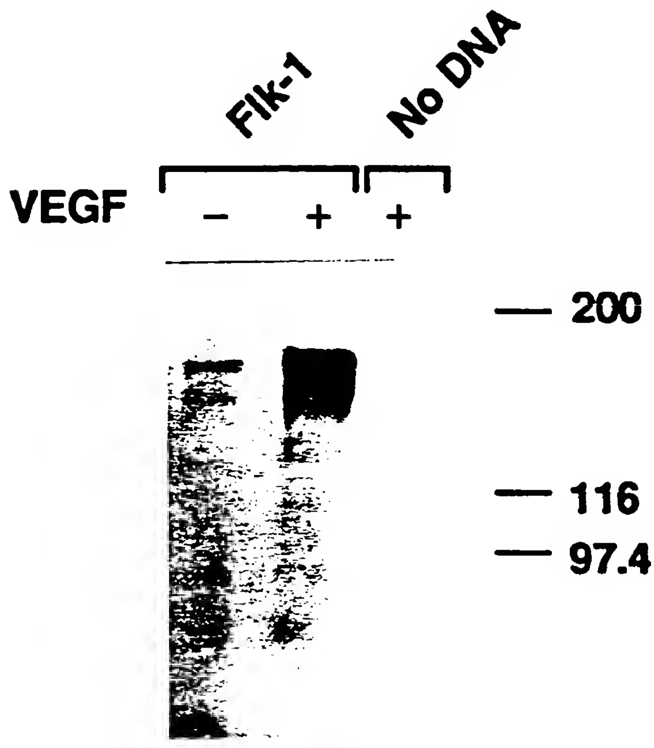

FIG. 10

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1    TATAGGGCGAATTGGGTACCGGGCCCCCCCTCGAGGTCGACGGTATCGATAAGCTTGATATCGAATTCGGGCCCAGACTGTGTCCCGCAGC   90

91   CGGGATAACCTGGCTGACCCCGATTCCGCGGACACCCGCTGACAGCCCCGGCTGGAGCCAGGGCGCCCGGTGCCCCCGCGCTCTCCCCCGGTCTT   160

181  GCGCTGCGGGGGCCATACCGCCTCTGTGACTTCTTTGCGGGCCAGGCACGGAGAAGGAGTCTGTGCCTGAGAAACTGGGCTCTGTGCCCA   270

                         M  E  S  K  A  L  L  A  V  A  L  W  F  C  V  H  T  R  A  A  S  V  G  L  T    25
271  GCGCGCGAGGTGCAGGATGGAGAGCAAGGCGCTGCTAGCTGTCGCTCTGTGGTTCTGCGTGCACACCCGAGCCGCCTCTGTGGGTTTGACT   360

26   G  D  F  L  H  P  P  K  L  S  T  Q  K  D  I  L  T  I  L  A  N  T  T  L  Q  I  T  C  R  G    55
361  GGCGATTTTCTCCATCCCCCCAAGCTCAGCACACAGAAAGACATACTGACAATTTTGGCAAATACAACCCTTCAGATTACTTGCAGGGGA   450

56   Q  R  D  L  D  K  L  M  P  N  A  Q  R  D  S  E  H  R  V  L  V  T  E  C  G  G  G  D  S  I    85
451  CAGCGGGACCTGGACAAGCTGATGCCCAATGCTCAGCGTGATTCTGAGCACAGGGTATTGGTGACTGAATGCGGCGGTGGTGACAGTATC   540

86   F  C  K  T  L  T  I  P  R  V  V  G  N  D  T  G  A  Y  K  C  S  Y  R  D  V  D  I  A  S  T    115
541  TTCTGCAAAACACTCACCATTCCCAGGGTGGTTGGAAATGATACTGGAGCCTACAAGTGCTCGTACCGGGACGTCGACATAGCCTCCACT   630

116  V  Y  V  Y  V  R  D  Y  R  S  P  F  I  A  S  V  S  D  Q  H  G  I  V  Y  I  T  E  N  K  N    145
631  GTTTATGTCTATGTTCGAGATTACAGATCACCATTCATCGCCTCTGTCAGTGACCAGCATGGCATCGTGTACATCACCGAGAACAAGAAC   720

146  K  T  V  V  I  P  C  H  G  S  I  S  N  L  N  V  S  L  C  A  R  Y  P  E  K  R  F  V  P  D    175
721  AAAACTGTGGTGATCCCCTGCCACGGGTCGATTTCAAACCTCAATGTGTCTCTTTGCGCTAGGTATCCAGAAAAGAGATTTGTTCCGGAT   810

176  G  N  R  I  S  K  D  S  H  I  G  F  T  L  P  S  Y  M  I  S  Y  A  G  M  V  F  C  E  A  K    205
811  GGAAACAGAATTTCCAAGGACAGCCACATAGGCTTTACTCTCCCCAGTTACATGATCAGCTATGCCGGCATGGTCTTCTGTGAGGCAAAG   900

206  I  N  D  K  T  Y  Q  S  I  M  Y  I  V  V  V  V  G  Y  R  I  Y  D  V  I  L  S  P  P  H  H    235
901  ATCAATGATAAAACCTATCAGTCTATCATGTACATAGTTGTGGTTGTAGGATATAGGATTTATGATGTGATTCTGAGCCCCCCGCATCAT   990

236  I  K  L  S  A  G  K  K  L  V  L  N  C  T  A  R  T  E  L  N  V  G  L  D  F  T  M  H  S  P    265
991  ATTAAGCTATCTGCCGGAAAGAAACTTGTCTTAAATTGTACAGCGAGAACAGAGCTCAATGTGGGGCTTGATTTCACCTGGCACTCTCCA   1080

266  P  S  K  S  H  H  K  K  I  V  N  R  D  V  K  P  F  P  G  T  V  A  K  M  F  L  S  T  L  T    295
1081 CCTTCAAAGTCTCATCATAAGAAGATTGTAAACCGGGATGTGAAACCCTTTCCTGGGACTGTGGCGAAGATGTTTTTGAGCACCTTGACA   1170

296  I  E  S  V  T  K  S  D  Q  G  E  Y  T  C  V  A  S  S  G  R  M  I  K  R  N  R  T  F  V  R    325
1171 ATAGAAAGTGTGACCAAGAGTGACCAAGGGGAATACACCTGTGTAGCGTCCAGTGGACGGATGATCAAGAGAAATAGAACATTTGTCCGA   1260
```

FIG.11A

```
326  V H T K P F I A F G S G M K S L V E A T V G S Q V R I P V K  355
1261 GTTCACACAAAGCCTTTTATTGCTTTCGGTAGTGGGATGAAATCTTTGGTGGAAGCCACAGTGGGCAGTCAAGTCCGAATCCCTGTGAAG 1350

356  Y L S Y P A P D I K N Y R N G R P I E S N Y T M I V G D K L  385
1351 TATCTCAGTTACCCAGCTCCTGATATCAAATGGTACAGAAATGGAAGGCCCATTGAGTCCAACTACACAATGATTGTTGGCGATGAACTC 1440

386  T I M K V T K R D A Q N Y T V I L T N P I S N E K Q S H M V  415
1441 ACCATCATGGAAGTGACTGAAAGAGATGCAGGAAACTACACGGTCATCCTCACCAACCCCATTTCAATGGAGAAACAGAGCCACATGGTC 1530

416  S L V V K V P P Q I G E K A L I S P M D S Y Q Y G T M Q Y L  445
1531 TCTCTGGTTGTGAATGTCCCACCCCAGATCGGTGAGAAAGCCTTGATCTCGCCTATGGATTCCTACCAGTATGGGACCATGCAGACATTG 1620

446  T C T V Y A N P P L H H I Q N Y N Q L E E A C S Y R P G Q T  475
1621 ACATGCACAGTCTACGCCAACCCTCCCCTGCACCACATCCAGTGGTACTGGCAGCTAGAAGAAGCCTGCTCCTACAGACCCGGCCAAACA 1710

476  S P Y A C K E K R H V E D F Q G G N K I E V T K N Q Y A L I  505
1711 AGCCCGTATGCTTGTAAAGAATGGAGACACGTGGAGGATTTCCAGGGGGGAAACAAGATCGAAGTCACCAAAAACCAATATGCCCTGATT 1800

506  K G K N K T V S T L V I Q A A N V S A L Y K C E A I N K A G  535
1801 GAAGGAAAAAACAAAACTGTAAGTACGCTGGTCATCCAAGCTGCCAACGTGTCAGCGTTGTACAAATGTGAGCCATCAACAAAGCGGGA 1890

536  R G E R V I S F H V I R G P E I T V Q P A A Q P T E Q E S V  565
1891 CGAGGAGAGAGGGTCATCTCCTTCCATGTGATCAGGGGTCCTGAAATTACTGTGCAACCTGCTGCCCAGCCAACTGAGCAGGAGAGTGTG 1980

566  S L L C T A D R N T F E N L T N Y K L G S Q A T S V H N G F  595
1981 TCCCTGTTGTGCACTGCAGACAGAAATACGTTTGAGAACCTCACGTGGTACAAGCTTGGCTCACAGGCAACATCGGTCCACATGGGCGAA 2070

596  S L T P V C K N L D A L N K L M G T M F S N S T N D I L I V  625
2071 TCACTCACACCAGTTTGCAAGAACTTGGATGCTCTTTGGAAACTGAATGGCACCATGTTTTCTAACAGCACAAATGACATCTTGATTGTG 2160

626  A F Q N A S L Q D Q G D Y V C S A Q D K K T K K R H C L V K  655
2161 GCATTTCAGAATGCCTCTCTGCAGGACCAAGGCGACTATGTTTGCTCTGCTCAAGATAAGAAGACCAAGAAAAGACATTGCCTGGTCAAA 2250

656  Q L I I L K R M A P H I T G N L S N Q T T T I Q E T I H V T  685
2251 CAGCTCATCATCCTAGAGCGCATGGCACCCATGATCACCGGAAATCTGGAGAATCAGACAACAACCATTGGCGAGACCATTGAAGTGACT 2340

686  C P A S C N P T P N I T K F K D N E T L V E D S G I V L R D  715
2341 TGCCCAGCATCTGGAAATCCTACCCCACACATTACATGGTTCAAAGACAACGAGACCCTGGTAGAAGATTCAGGCATTGTACTGAGAGAT 2430

716  G N R N L T I R R V R K E D G G L Y T C Q A C N V L G C A R  745
2431 GGGAACCGGAACCTGACTATCCGCAGGGTGAGGAAGGAGGATGGAGGCCTCTACACCTGCCAGGCCTGCAATGTCCTTGGCTGTGCAAGA 2520
```

FIG.11B

SUBSTITUTE SHEET

746 A E T L F I I E G A Q H K T N L E V I I L V G T A V I A M F 775
2521 GCGGAGACCCTCTTCATAATAGAAGGTGCCCAGGAAAAGACCAACTTGGAAGTCATTATCCTCGTCGGCACTGCAGTGATTGCCATGTTC 2610

776 F W L L L V I V L R T V K R A N H G K L K T G Y L S I V M D 805
2611 TTCTGGCTCCTTCTTGTCATTGTCCTACGGACCGTTAAGCGGGCCAATCACGGGAAGCTGAAGACAGGCTACTTGTCTATTGTCATGGAT 2700

806 F D K L P L D H R C K E L P Y D A S K N E F P R D R L K L G 835
2701 CCAGATGAATTGCCCTTGGATGAGCGCTGTGAACGCTTGCCTTATGATGCCAGCAAGTGGGAATTCCCCAGGGACCGGCTGAAACTAGGA 2790

836 K F L G R G A F G Q V I E A D A F G I D K T A T C K T V A V 865
2791 AAACCTCTTGGCCGCGGTGCCTTCGGCCAAGTGATTGAGGCAGACGCTTTTGGAATTGACAAGACAGCGACTTGCAAAACAGTAGCCGTC 2880

866 K M L K E G A T H S E H R A L M S K L K I L I H I G H H L N 895
2881 AAGATGTTGAAAGGAGGAGCAACACACAGCGAGCATCGAGCCCTCATGTCTGAACTCAAGATCCTCATCCACATTGGTCACCATCTCAAT 2970

896 V V N L L G A C T K P G G P L M V I V E F C K F G N L S T Y 925
2971 GTGGTGAACCTCCTAGGCGCCTGCACCAAGCCGGGAGGGCCTCTCATCGTGATTGTCGAATTCTGCAAGTTTGGAAACCTATCAACTTAC 3060

926 L E G K R N E F V P Y K S K G A R F R Q C K D Y V G K L S V 955
3061 TTACGGGGCAAGAGAAATGAATTTGTTCCCTATAAGAGCAAAGGGGCACGCTTCCGCCAGGGCAAGGACTACGTTGGGGAGCTCTCCGTG 3150

956 D L K R R L D S I T S S Q S S A S S G F V K H K S L S D V E 985
3151 GATCTGAAAAGACGCTTGGACAGCATCACCAGCAGCCAGAGCTCTGCCAGCTCAGGCTTTGTTGAGGAGAAATCGCTCAGTGATGTAGAG 3240

986 K K K A S K K L Y K D F L T L K H L I C Y S F Q V A K G M E 1015
3241 GAAGAAGAAGCTTCTGAAGAACTGTACAAGGACTTCCTGACCTTGGAGCATCTCATCTGTTACAGCTTCCAAGTGGCTAAGGGCATGGAG 3330

1016 F L A S R K C I H R D L A A R N I L L S E K N V V K I C D F 1045
3331 TTCTTGGCATCAAGGAAGTGTATCCACAGGGACCTGGCAGCACGAAACATTCTCCTATCGGAGAAGAATGTGGTTAAGATCTGTGACTTC 3420

1046 G L A R D I Y K D P D Y V R K G D A R L P L K W M A P E T I 1075
3421 GGCTTGGCCCGGGACATTTATAAAGACCCGGATTATGTCAGAAAAGGAGATGCCCGACTCCCTTTGAAGTGGATGGCCCCGGAAACCATT 3510

1076 F D R V Y T I Q S D V W S F G V L L W E I F S L G A S P Y P 1105
3511 TTTGACAGAGTATACACAATTCAGAGCGATGTGTGGTCTTTCGGTGTGTTGCTCTGGGAAATATTTTCCTTAGGTGCCTCCCCATACCCT 3600

1106 G V K I D E E F C R R L K E G T R M R A P D Y T T P E M Y Q 1135
3601 GGGGTCAAGATTGATGAAGAATTTTGTACGACATTGAAAGAAGGAACTAGAATGCGGGCTCCTGACTACACTACCCCAGAAATGTACCAG 3690

FIG.11C

1136 T M L D C N H E D P N Q R P S F S E L V E H L G N L L Q A N 1165
3691 ACCATGCTGGACTGCTGGCATGAGGACCCCAACCAGAGACCCTCGTTTTCAGAGTTGGTGGAGCATTTGGGAAACCTCCTGCAAGCAAAT 3780

1166 A Q Q D G K D Y I V L P M S E T L S M K E D S G L S L P T S 1195
3781 GCCCAGCAGGATGGCAAAGACTATATTGTTCTTCCAATGTCAGAGACACTGAGCATGGAAGAGGATTCTGGACTCTCCCTGCCTACCTCA 3870

1196 P V S C M E E E H V C D P K Y H Y D N T A G I S H Y L Q N S 1225
3871 CCTGTTTCCTGTATGGAGGAAGAGGAAGTGTGCGACCCCAAATTCCATTATGACAACACAGCAGGAATCAGTCATTATCTCCAGAACAGT 3960

1226 K R K S R P V S V K T F H D I P L E E P E V K V I P D D S Q 1255
3961 AAGCGAAAGAGCCGGCCAGTGAGTGTAAAAACATTTGAAGATATCCCATTGGAGGAACCAGAAGTAAAAGTGATCCCAGATGACAGCCAG 4050

1256 T D S G M V L A S E E L K T L E D R N K L S P S F G G M M P 1285
4051 ACAGACAGTGGGATGGTCCTTGCATCAGAAGAGCTGAAAACTCTGGAAGACAGGAACAAATTATCTCCATCTTTTGGTGGAATGATGCCC 4140

1286 S J S R E S V A S E G S B Q T S G T Q S G T G S D D T D T T 1315
4141 AGTAAAAGCAGGGAGTCTGTGGCCTCGGAAGGCTCCAACCAGACCAGTGGCTACCAGTCTGGGTATCACTCAGATGACACAGACACCACC 4230

1316 V Y S S D E A C L L K M V D A A V H A D S G T T L Q L T S C 1345
4231 GTGTACTCCAGCGACGACCCAGGACTTTTAAAGATGGTGGATGCTGCAGTTCACGCTGACTCAGGGACCACACTGCAGCTCACCTCCTGT 4320

1346 L N G S G P V P A P P P T P G N H E R G A A * 1367
4321 TTAAATGGAAGTGGTCCTGTCCCGGCTCCGCCCCCAACTCCTGGAAATCACGAGAGAGGTGCTGCTTAGATTTTCAAGTGTTGTTCTTTC 4410

4411 CACCACCCGGAAGTAGCCACATTTGATTTTCATTTTTGGAGGAGGGACCTCAGACTGCAAGGAGCTTGTCCTCAGGGCATTTCCAGAGAA 4500

4501 GATGCCCATGACCCAAGAATGTGTTGACTCTACTCTCTTTTCCATTCATTTAAAAGTCCTATATAATGTGCCCTGCTGTGGTCTCACTAC 4590

4591 CAGTTAAAGCAAAAGACTTTCAAACACGTGGACTCTGTCCTCCAACAAGTGGCAACGGCACCTCTGTGAAACTGGATCGAATGGGCAATG 4680

4681 CTTTGTGTGTTGAGGATGGGTGAGATGTCCCAGGGCCGAGTCTGTCTACCTTGGAGGCTTTGTGGAGGATGCGGGCTATGAGCCAAGTGT 4770

4771 TAAGTGTGGGATGTGGACTGGGAGGAAGGAAGGCGCAAGTCGCTCGGAGAGCGGTTGGAGCCTGCAGATGCATTGTGCTGGCTCTGGTGG 4860

4861 AGGTGGGCTTGTGGCCTGTCAGGAAACGCAAAGGCGGCCGCCAGGGTTTGGTTTTGGAAGGTTTGCGTGCTCTTCACAGTCGGGTTACAG 4950

4951 GCGAGTTCCCTGTGGCGTTTCCTACTCCTAATGAGAGTTCCTTCCGGACTCTTACGTGTCTCCTGGCCTGGCCCCAGGAAGGAAATGATG 5040

5041 CAGCTTGCTCCTTCCTCATCTCTCAGGCTGTGCCTTAATTCAGAACACCAAAAGAGAGGAACGTCGGCAGAGGCTCCTGACGGGGCCGAA 5130

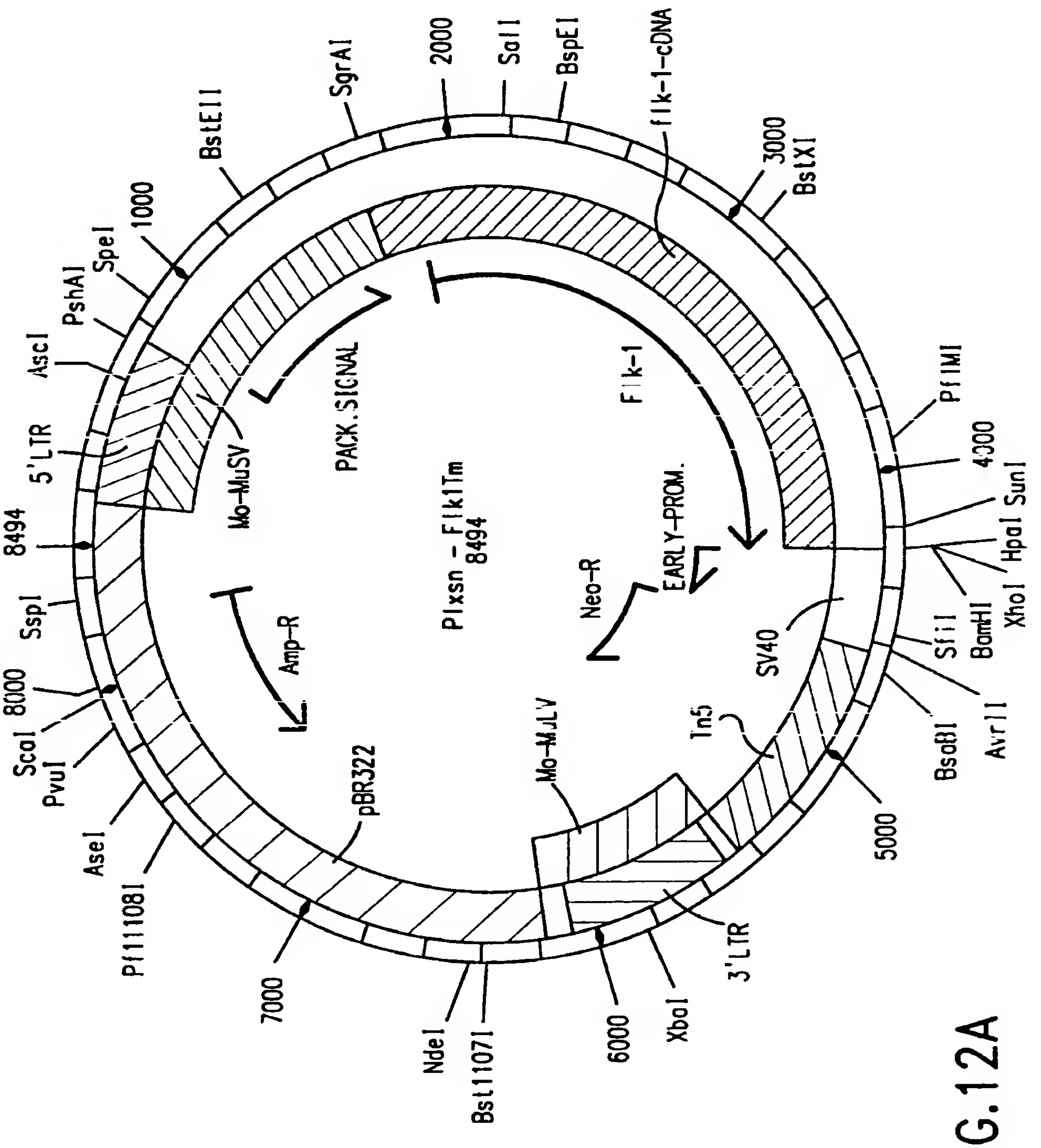
5131 GAATTGTGAGAACAGAACAGAAACTCAGGGTTTCTGCTGGGTGGAGACCCACGTGGCGCCCTGGTGGCAGGTCTGAGGGTTCTCTGTCAA 5220

5221 GTGGCGGTAAAGGCTCAGGCTGGTGTTCTTCCTCTATCTCCACTCCTGTCAGGCCCCCAAGTCCTCAGTATTTTAGCTTTGTGGCTTCCT 5310

5311 GATGGCAGAAAAATCTTAATTGGTTGGTTTGCTCTCCAGATAATCACTAGCCAGATTTCGAAATTACTTTTTAGCCGAGGTTATGATAAC 5400

5401 ATCTACTGTATCCTTTAGAATTTTAACCTATAAAACTATGTCTACTGGTTTCTGCCTGTGTGCTTATGTT 5470

FIG.11D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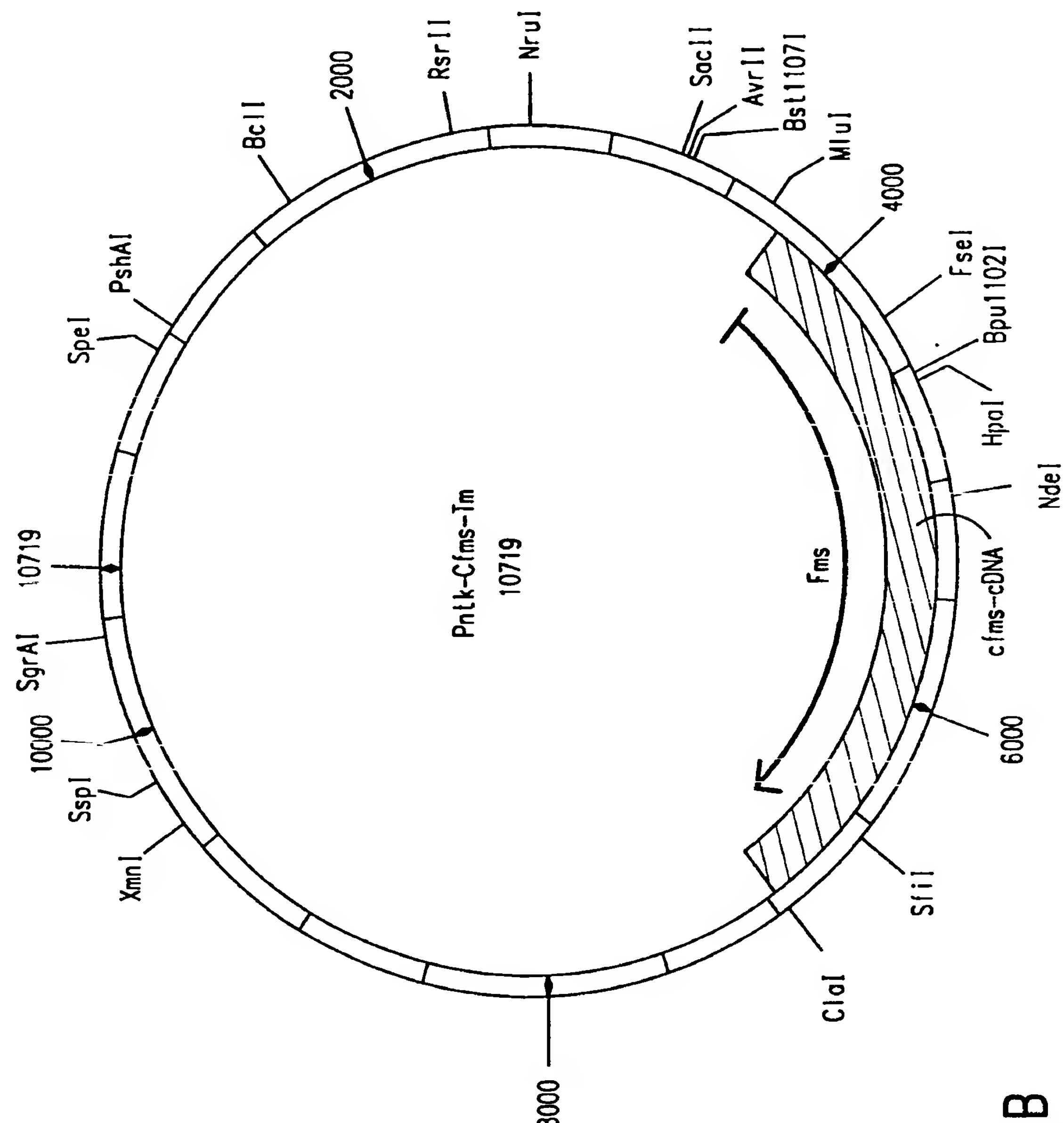
SalI
BspEI
flk-1-cDNA
3000
BstXI
SgrAI
BstEII
1000
SpeI
PshAI
AscI
5'LTR
8494
SspI
8000
ScaI
PvuI
AseI
Pfl1108I
7000
NdeI
Bst1107I
6000
XbaI
3'LTR
5000
BsaBI
AvrII
SfiI
BamHI
XhoI
HpaI
SunI
4000
PflMI
Mo-MuSV
PACK.SIGNAL
Flk-1
EARLY-PROM.
Neo-R
SV40
Tn5
Mo-MuLV
pBR322
Amp-R

FIG.12A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Fms
cfms-cDNA
NruI
RsrII
2000
BclI
PshAI
SpeI
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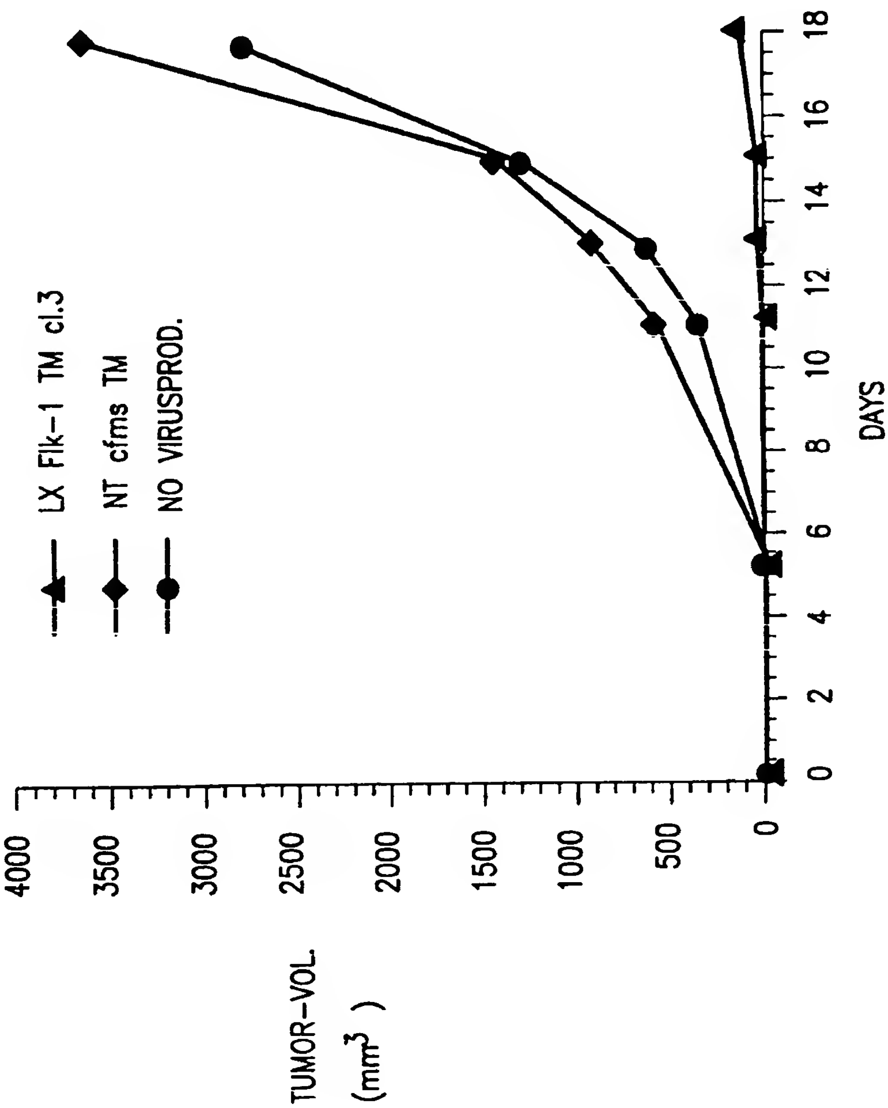
SgrAI
10000
SspI
XmnI
8000
ClaI
SfiI
6000
NdeI
HpaI
Bpu1102I
FseI
4000
MluI
Bst1107I
AvrII
SacII

FIG.12B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3500
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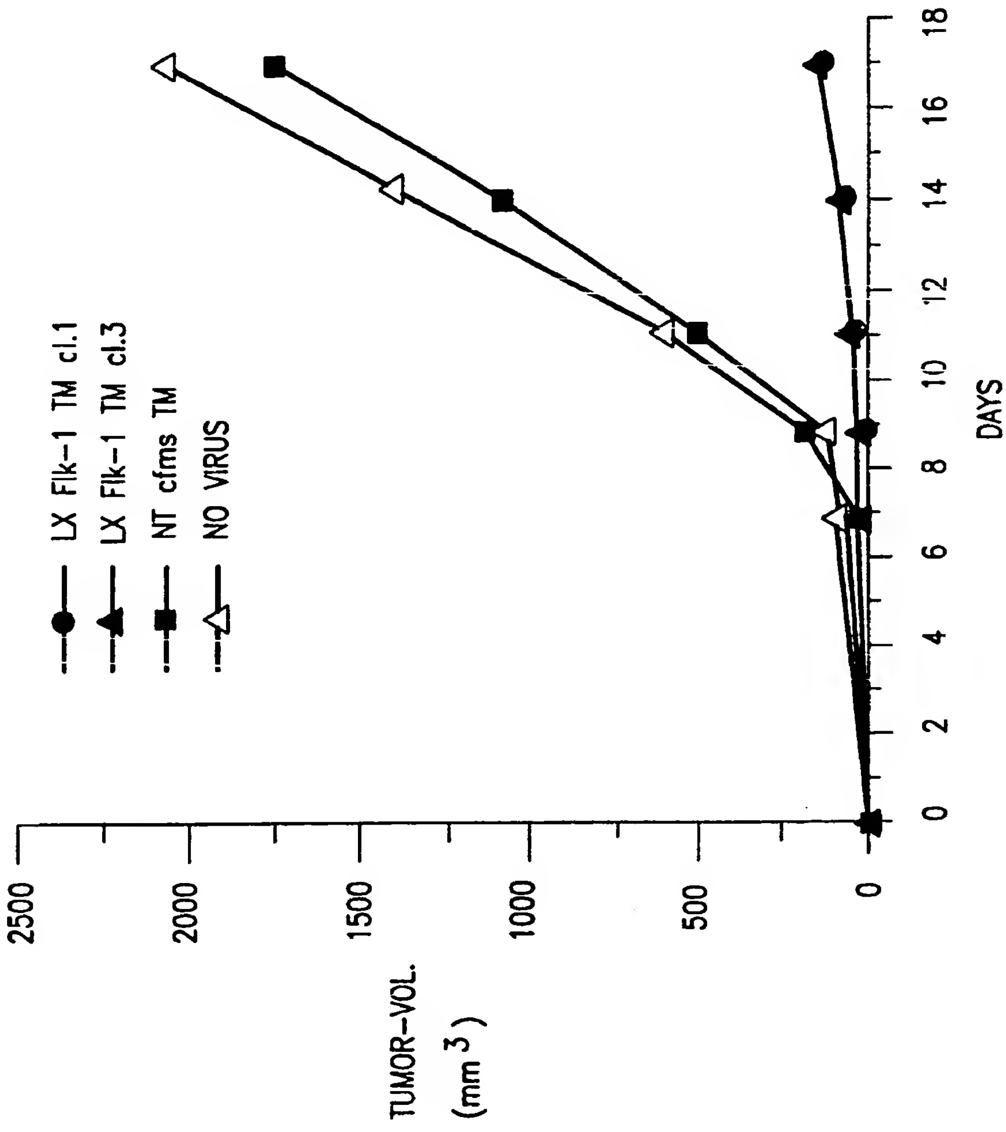
500
0
0
2
4
6
8
10
12
14
16
18
DAYS

FIG.13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NO VIRUS
TUMOR-VOL.
(mm 3)
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2000
1500
1000
500
0
0
2
4
6
8
10
12
14
16
18
DAYS

FIG.14

INTERNATIONAL SEARCH REPORT

International Application No
P[illegible]EP 93/03191

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C12N15/12 C07K13/00 C12P21/08 C12N15/86 C12Q1/68
G01N33/567 A61K37/02 C12N15/62

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C07K C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 88 , October 1991 , WASHINGTON US pages 9026 - 9030 MATTHEWS, W. ET AL.; 'A receptor tyrosine kinase cDNA isolated from a population of enriched primitive hematopoietic cells and exhibiting close genetic linkage to c-kit.' see the whole document	1-11
X Y	WO,A,92 17486 (TRUSTEES OF PRINCETON UNIVERSITY, US) 15 October 1992 see the whole document	1-11, 14-37 12,13, 38,39,41

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

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Date of the actual completion of the international search	Date of mailing of the international search report
11 March 1994	15 -04- 1994

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Nauche, S

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO,A,92 03459 (SLOAN KETTERING INSTITUTE OF CANCER, US) 5 March 1992 see the whole document ---	12,13
Y	BIOTECHNOLOGY vol. 3, no. 8 , August 1985 , NEW YORK US pages 689 - 693 MC CORMICK, D.; 'Human gene therapy : the first round' see the whole document ---	38,39,41
P,X	CELL vol. 72 , 26 March 1993 , CAMBRIDGE, NA US pages 835 - 846 MILLAUER, B., WIZIGMANN-VOOS, S., SCHNURCH, H., MARTINEZ, R., MOLLER, N.P., RISAU, W., AND ULLRICH, A.; 'High affinity VEGF binding and developmental expression suggest Flk-1 as a major regulator of vasculogenesis and angiogenesis.' see the whole document -----	1-43

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Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark : Although claims 25-28, 31-35, 43 are directed to a method of treatment of the human/animal body as well as diagnostic methods (Rule 39.1 (iv) PCT) the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 93/03191

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9217486	15-10-92	US-A- 5185438	09-02-93
		AU-A- 1924892	02-11-92
		CA-A- 2107463	03-10-92
		EP-A- 0580760	02-02-94
		WO-A- 9300349	07-01-93
		US-A- 5283354	01-02-94
		US-A- 5270458	14-12-93
		AU-A- 2296292	25-01-93
		AU-A- 3139493	15-06-93
		WO-A- 9310136	27-05-93
WO-A-9203459	05-03-92	AU-A- 8510691	17-03-92
		CA-A- 2090469	28-02-92
		EP-A- 0546054	16-06-93